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(54) Title: METHODS AND VECTORS FOR TRANSFORMATION OF PLANT CELLS

(57) Abstract

Vectors and methods suitable for both direct and *Agrobacterium*-mediated transformation of plants. The vectors comprise easily manipulated expression cassettes and optionally contain border sequences associated with *Agrobacterium* plasmid DNA transfer. By using an intermediate carrier vector which contains the requisite expression control sequences in accessible form, a wide variety of single and multiple cassette vectors can be constructed.

-1-

METHODS AND VECTORS FOR TRANSFORMATION
OF PLANT CELLS

Technical Field

The present invention relates to the field of
5 application of recombinant DNA techniques to the
transformation of higher plants. More specifically, the
invention relates to vectors useful in transforming
higher plants and in conferring desired properties on
them, and to methods of employing such vectors in
10 transformations.

Background Art

After nearly a decade of increasing
sophistication in the field of recombinant DNA
technology, application of recombinant techniques to
15 modification of the genetic structure of higher plants
appears to be the most resistant to predictability and
even threshold accomplishment of desired results. It has
been possible for some time to transform prokaryotes and
to convey to them desirable properties relating to
20 manufacture of foreign proteins; more recently,
eukaryotic microorganisms, such as yeast, have been
successfully transformed to adapt them to desirable
characteristics, and, perhaps most recently, vertebrate
cells in tissue culture have been employed as host cells
25 for transformation with recombinant expression vectors.
However, although there have been some successes,
techniques for succ ssful transformation of plant cells

-2-

have remained elusive. It has not even been possible to
maintain plant-derived cell lines in "tissue culture"
conditions and then regenerate intact plants except for a
few species of plants, notably tobacco, petunias, and
5 cotton.

Successful, reproducible transformation of plant
cells, combined with subsequent regeneration of the plant
tissue into "normal" plants to allow these to function
according to the normal agrarian reproductive cycle
10 carries the possibility of some highly desirable results.
For example, plants transformed with genes which, when
expressed, would confer resistance upon the plant to
predation by insects, to weed killers, or to infection by
plant diseases would result in a far more efficient
15 agricultural and forestation system. Accordingly,
straightforward ways to assure the incorporation of
foreign genetic material into plant tissues on a
permanent basis have long been sought.

Possibly because of the rigidity and limited
20 permeability of plant cell walls, direct transformation
of plant cells with "naked" DNA has proved impossible.
Even after removal of the cell walls, and culturing of
the resultant protoplasts, it appears that plant cells
are refractory to integration of foreign naked DNA.
25 Thus, efforts have focused on co-opting methods for DNA
insertion which already exist in nature. Notable among
these is the capacity of Agrobacterium tumefaciens to
infect dicotyledonous plants and to confer on them genetic
alterations which result in the growth of what is
30 commonly known as crown gall tumor.

Some factors associated with the integration of
Agrobacterium DNA into infected plants are now
understood. First, it is clear that the bacterial DNA

-3-

transforming the infected plant cell is non-chromosomal
DNA; and is transferred from a plasmid vector (Ti).

At least two Ti (tumor inducing) plasmid types
(which are designated according to the opines associated
5 with them) have been mapped and studied extensively:
"nopaline" Ti plasmids such as pTiT37 (Depicker, A., et
al, J Molec Appl Genet (1982) 1:561; Bevan, M., et al,
Nucleic Acids Res (1983) 11:369) and pTiC58 (see, e.g.,
Shaw, C. H., et al, Gene (1983) 23:315); and "octopine"
10 plasmids such as pTiB6S3 (DeGreve, H., et al, J Molec
Appl Genet (1982) 1:499). They are quite large (e.g.,
132 Mdal or about 200 kilobases (kb) for pTiC58) and have
a number of relevant features. There is, in each case, a
"virulence" (vir) region which appears to be associated
15 with those functions permitting successful infection and
integration of certain DNA portions into the host plant
cell, i.e., this region is associated with encoding
whatever products effect the attachment of the bacterium
to a plant cell, transfer of the plasmid DNA into the
20 plant cell, or other necessary functions required for
infection. A second region of significance is the
"transfer-DNA" or T-DNA region constituting that portion
of the plasmid which, after infection, actually becomes
integrated into the plant genome.
25   In a wild type Ti plasmid, this T-region has a
number of important characteristics. First, it generally
contains an opine synthase region which, when
expressed, provides the enzymes required for the
synthesis of a characteristic opine--nopaline in the case
30 of the nopaline-type plasmids, and octopine in the case
of octopine-type plasmids. (Nopaline is a conjugate of
arginine and alpha ketoglutaric acid; octopine is a
conjugate of arginine and pyruvic acid.) Presumably,
synthesis of these opines serves a natural function in

-4-

the normal course of infecti n--an additional region of
the plasmid encodes enzymes which permit the degradation
and metabolism of the particular opine encoded by the
synthase in the T-DNA region. Thus, plant cells
5 infected by a particular plasmid acquire the ability to
synthesize a particular opine, i.e., nopaline or
octopine, which product then is a nutrient for additional
infecting cells. That is, the ability to metabolize a
particular opine is encoded on the same plasmid as the
10 synthase encoding region, but not integrated into the
host cells' genome.
        The T-DNA region also encodes genes which
appear to affect endogenous levels of the plant hormones
cytokinin and auxin. This is doubly significant. On the
15 positive side, this function is useful in selecting
successful transformants in tissue culture, since
generally only transformed cells have the capacity to
synthesize these hormones and are capable of reproduction
in their absence from the medium. On the other hand, the
20 presence of these specific genes in the transferred DNA
has natural consequences in the course of infection, and
because of the increased and unbalanced amounts of plant
hormones, transformed plant cells fail to regenerate into
complete normal plants and infected intact plants develop
25 crown galls. Indeed, it has been shown that if specific
mutations are made in the T-DNA regions affecting the
levels of these hormones, sufficient imbalance of the
auxin-cytokinin ratio can be obtained to result in growth
predominantly of roots or predominantly of shoots from
30 the resulting tumor tissue.
        Finally, the T-DNA is flanked by "border"
sequences which contain approximately 25 nucleotide
direct repeats. Because of the nature of these
sequences, it is surmised, although not known, that these

-5-

regions may be involved in the insertion of the
intervening T-DNA into the chromosome of the infected
cell.

5   Because the T-DNA region is integrated into the
host cell chromosome, attempts have been made to utilize
this region as a vehicle for transformation of plant host
cells by foreign DNA. None of these attempts have been
entirely successful, if evaluated for the goal of
obtaining the desired combination of an expressed foreign
10 gene in the context of a normal plant.

Shaw, C. H., et al, Gene (1983) 23:315
succeeded in transforming an axenic culture of tobacco
seedlings by infecting them with A. tumefaciens
presumably containing a recombined Ti plasmid. This
15 plasmid was thought to result from an in vivo cross-over
in the A. tumefaciens cells involving homologous regions
of the T-DNA. The putative recombined vector was derived
from a broad-host range intermediate vector containing
the HindIII-23 right-end T region fragment of the
20 nopaline plasmid pTiC58 into which the rabbit β-globin
gene had been inserted, and the Ti plasmid (also pTiC58)
indigenous to the Agrobacterium. The infected seedlings
incorporated the β-globin DNA, but did not make any
transcripts of it.

25   Herrera-Estrella, L., et al, Nature (1983)
303:209 succeeded in obtaining expression of the coding
sequence for a heterologous protein conferring
chloramphenicol resistance in axenic cultures derived from
Agrobacterium infection induced-tobacco seedling tumors.
30 The coding sequence for chloramphenicol acetyl
transferase (CAT) was linked to the promoter sequences of
the nopaline synthase gene to obtain a NOS-CAT
chimaeric gene and placed in a recombinant vector with a
narrow host range (E. coli) origin of replication, a

-6-

region of T-DNA homology, and the ampicillin resistance
conferring (Amp^R) sequences. This plasmid was
transformed into A. tumefaciens harboring pTiC58 and
successful transformants selected for Amp^R, thus insuring
5 that recombination between the bacterial vector and Ti
plasmid had occurred. When the Agrobacterium containing
the presumed recombined plasmid was used to infect
tobacco seedlings, the resulting tumor cells, grown in
tissue culture were not regenerated into plants, but were
10 able to produce the CAT-encoded protein.

          Fraley, R. T., et al, Proc Natl Acad Sci
(USA) (1983) 80:4803 disclose transformation of
petunia protoplasts by co-cultivation with A. tumefaciens
selected, analogously to the work of the preceding
15 paragraph, for an in vivo recombination of two vectors:
the first a narrow host range plasmid vector containing a
chimaeric gene having a coding sequence for a protein
conferring neomycin resistance linked to the nopaline
synthase promoter, and having a sequence homologous to
20 pTiB6S3 to encourage recombination, and the second
indigenous Ti plasmid pTiB6S3. Protoplasts were obtained
which were resistant to the aminoglycoside antibiotic,
kanamycin, normally toxic to plant cells. It was assumed
that resistance is conferred by the inserted gene.
25 Normal plants were not regenerated.

          It has also been shown by de Framond, A. J.,
Biotechnology (1983) 262-269, and by Hoekema, A., et al,
Nature (1983) 303:179 that the virulence function and
T-DNA functions of the Ti plasmid can be separated into a
30 bipartite system which will cotransform plant cells upon
infection and successfully produce tumors.

          Finally, Barton, K. A., et al, Cell (1983)
32:1033 discloses experiments which show that the DNA
sequence of alcohol dehydrogenase (ADH) from yeast

-7-

inserted into the "rooty" locus of a Ti plasmid is found
in multiple copies in DNA isolated from plants
regenerated after infection, although the cells are not
tumorous--i.e., they required added cytokinin for growth.
5 Seeds obtained from self pollination of these plants also
generated normal tobacco plants, except, apparently, for
the inclusion of approximately 20 copies of the ADH
coding sequence in their DNA. The ADH was not expressed.
As the plants remain normal after the transformation with
10 respect to tumor formation, the vector used for
transformation was referred to as "disarmed".
          In none of the foregoing disclosures has it
been possible to show both expression and regeneration of
normal plants in the same experiment. The present
15 invention provides a means to obtain this desirable
result. It also offers greater control over the nature
of the expression obtained by permitting standard
recombinant DNA techniques to be employed in constructing
successful transformation vectors without the necessity
20 for selecting for an <u>in vivo</u> recombination event.

Disclosure of the Invention
          The present invention in one aspect relates to
vectors useful for expression of foreign gene sequences
in plant tissues. A basic intermediate carrier vector
25 behaves as a receiving unit which permits the
introduction of any desired foreign gene sequence so as
to be suitably disposed to take advantage of promoter and
polyadenylation signal sequences which are operable in
plant tissues. A wide variety of foreign genes can be
30 introduced with facility. Further, when such desired gene
sequences have been inserted, the resulting expression
carrier vectors can be spliced using standard recombinant
DNA restriction and ligation techniques to contain any

desired number of expression cassettes for a variety of
genes. Among these cassettes, it may be desirable to
include a marker which will permit easy selection of
successfully transformed cells.

5   The foregoing expression carrier vectors, when
transformed directly into plant tissue, unlike the
recombined vectors employed in the standard Ti vector
transformation techniques, do not produce tumors, and
plant cells transformed with these carrier vectors alone
10 are capable both of normal regeneration and of expression
of the desired gene.

           Accordingly, in one aspect, the present
invention relates to expression carrier vectors effective
in expressing the coding sequence of foreign genes in
15 recombinant plant cells and to the intermediate DNA
sequences and vectors useful in constructing them. Such
expression carrier vectors include:

           1. Recombinant vectors containing one or more
expression cassettes, each such expression cassette
20 comprising a promoter normally operable in plant cells
and a polyadenylation signal operable in plant cells,
both operably linked to a coding sequence for a desired
foreign protein. The expression carrier vectors may
contain more than one such expression cassette. These
25 vectors also contain at least one cassette-unique
restriction site at the terminus of at least one
expression cassette. This class of vectors is useful in
obtaining the expression of the foreign coding sequences
in plant cells after direct transformation of such cells ·
30 with these vectors.

           2. Another class of expression carrier vectors
comprises those of the preceding paragraph, but modified
so as to contain at least one A. tumefaciens T-DNA border
sequence proximate a terminus of the expression cassette,

-9-

or if multiple expression cassettes are contained in the
vector, at a terminus of these series. Preferably, the
single expression cassette, or the series of expression
cassettes, is framed by two such T-DNA border sequences.
5 These vectors also contain, on their vector portions, a
broad host range bacterial origin of replication. They
are useful in transforming plant cells so as to obtain
expression of foreign protein, either by the direct
transformation methods employed in connection with the
10 vectors of the previous paragraph, or in transformation
methods which involve the mediation of the virulence
regions of an Agrobacterium Ti plasmid.

In other aspects, the invention relates to
intermediate plasmids and DNA sequences employed in the
15 construction of the foregoing vectors, to methods of
transforming plants or plant cells using these vectors,
to plants or plant cells transformed with such vectors,
to methods of producing foreign proteins using plants so
transformed, and to the proteins so produced.

20 Brief Description of the Drawings
Figure 1 shows the construction of pCMC59 and
pCMC60.
Figure 2 shows the construction of pCMC121.
Figure 3 shows the construction of pCMC101.
25 Figure 4 shows the construction of pCMC91.
Figure 5 shows the construction of pCMC72.

Modes of Carrying Out the Invention
A. Definitions
As used herein, a promoter sequence "normally
30 operable in plant cells" refers to a promoter sequence
which is not only coincidentally compatible with plant
cells in the sense of being minimally capable of

-10-

initiating transcription of a coding sequence into mRNA,
but which is found natively to effect gene expression in
plant tissue. Such promoter may be of plant origin;
however, more typically in the embodiments of the
5 invention it is, in fact, a bacterial sequence.
Ordinarily, the sequence may be relatively silent in the
bacterium and is designed to operate specifically in an
infected plant cell. For example, many of the vectors of
the present invention utilize the promoter for nopaline
10 synthase, which is carried on an _Agrobacterium tumefaciens_
plasmid, but which has not been reported to express in
the bacterium. However, when the plasmid is introduced
into infected plant cells, it operates in this context to
express the nopaline synthase coding sequence. These
15 promoters are, although not of plant origin, "normally
operable" therein.
        "Polyadenylation signal operable in plant
cells" refers to a sequence normally found 3' of a coding
sequence which appears to be necessary for its
20 transcription, transport of the polyA-mRNA from the
nucleus and its stable accumulation in the cytoplasm,
permitting subsequent translation in eukaryotic cells.
It is currently believed that the function of these
sequences is to signal polyadenylation of the messenger
25 RNA transcribed from the coding sequence. However, the
complete function of these signal sequences has not been
unequivocally established. Therefore, as used herein,
this term refers to those signals which follow the coding
sequence in DNA and assure its expression, including
30 translation, in the eukaryotic cellular environment. It
is not at present clear whether this ability to assure
expression in plants is confined to sequences which are
found to be so utilized by plant cells in nature.
Therefore, any sequence which in fact is successful in

-11-

performing this function in plant cells is includ d in
the definition.

"Polylinker" refers to a DNA sequence which
contains at least three closely associated restriction
5 enzyme sites after ligation into a receptor DNA sequence.

"Expression cassette" refers to a DNA sequence
which is capable of effecting expression of a contained
coding sequence in plants. Thus, a typical expression
cassette contains a promoter sequence normally operable
10 in plant cells, operably linked to the coding sequence
and a polyadenylation signal operable in plant cells also
operably linked to the coding sequence. The word
"cassette" is also used in connection with other DNA
sequences which are designed to be treated as a unit.
15 For example, a series of "expression cassettes" would
also be referred to as a "DNA sequence cassette".

"Cassette-unique" restriction site refers to an
endonuclease enzyme cleavage recognition site which is
found only once in the referenced cassette or series of
20 cassettes.

"Vector sequence" or "vector segment" or
"vector fragment" refers to DNA sequences on a plasmid
that are not part of an expression cassette, DNA sequence
cassette, or other "cassette" DNA sequence. Typical of
25 "vector sequences" are origins of replication and T-DNA
border sequences.

"Narrow host range bacterial origin of
replication" refers to an origin of replication which is
capable of function only in a very limited number of
30 bacterial species. Typically, in most recombinant work,
such replicons are confined to functionality in such
commonly used species as E. coli, and, in the context of
the present invention, fail to replicate in foreign hosts
such as Agrobacterium. Conversely, "broad host range

-12-

bacterial origin f replication" refers to those
replicons which are functional not only in their strain
of origin but also in disparate bacterial species.   In
the context of the present invention, the most meaningful
5 example would be those origins which are capable of
replication both in E. coli and in Agrobacterium.

As used herein, "T-DNA border sequences" refers
to repeating DNA sequences the same as, or analogous to,
those bordering the T-DNA portion of the Ti plasmids of
10 A. tumefaciens. In the wild type A. tumefaciens, these
repeating sequences frame the T-DNA which is transferred
into the chromosome of the infected cell. The definition
of border sequences as used in this invention is not
limited to these specific repeating segments, but
15 includes any modifications thereof which still are
functional in mediating the transfer of the intervening
material, with the aid of the virulence region of a Ti
plasmid, into a host cell genome. The abbreviations "LB"
(left border) and "RB" (right border) are used to
20 designate those sequences framing the T-DNA of the
A. tumefaciens plasmid.

"Plants" or "plant cells" refer to plant types
which are capable of reproduction by formation of seeds,
and these terms include the plant itself, the cells of
25 the plant, protoplasts which derived from plant cells,
other plant-derived cells such as tissue culture cells,
tumor cells, calli and seeds, and progeny thereof. It is
understood that progeny can undergo spontaneous or
intentional modifications when cultured over several
30 generations, and such modified progeny are intended to be
included as long as they are derived from the plant cell
or plant referred to.

"Operably linked" refers to sequences which are
juxtaposed in such a way that their functionality is

-13-

maintained. Thus, a promoter "operably linked" to a
coding sequences refers to a disposition of a promoter
which is capable of effecting the transcription and
translation of this sequence. Similarly, a
5 polyadenylation signal "operably linked" to a coding
sequence refers to a disposition of this signal which
permits production of protein from the coding sequence.

B. General Description and Preferred Embodiments
       The transformation techniques of the present
10 invention utilize a system of vector constructs which
permits facile manipulation of gene sequences in an
interchanageable set of vectors of general utility.

       B.1 The Intermediate Carrier Vector
       The seminal vector is an intermediate carrier
15 vector which is designed to permit convenient insertion
of any desired polypeptide encoding sequence.
       This vector comprises a "cassette" DNA sequence
which includes, in order, proceeding 5'-3' in the sense
strand:
20   (a) a first cassette-unique restriction site;
            (b) a promoter sequence (oriented 5'-3') which
is normally operable in plant cells;
            (c) a polylinker;
            (d) a polyadenylation signal (oriented 5'-3')
25 operable in plant cells; and
            (e) a second cassette-unique restriction site.
       The presence of the polylinker permits easy
manipulation in relation to coding sequences so as to
permit insertion either of an ATG-initiated sequence
30 encoding a "mature" protein or of a sequence into reading
frame with a partial coding sequence operably included
with the promoter.

-14-

Upon insertion of such coding sequences this
vector becomes an expression carrier vector capable of
expressing the coding sequence when transformed into
plant cells. Carrier vectors containing a wide variety
5 of coding sequences, for example, APH-I and APH-II (which
encode proteins conferring resistance to the
aminoglycoside antibiotic G418 or kanamycin, which are
toxic to yeast, plant and mammalian as well as to
bacterial cells); beta interferon, which may, in fact,
10 protect plants as well as mammals against viral infection
(Orchansky, P. et al, Proc Natl Acad Sci (USA) (1982)
79:2278; Bt-toxin which acts as an internal pesticide for
plant tissues; and CAT which catalyzes an acetylation
modification of chloramphenicol. This list is far from
15 exhaustive, and any coding sequence which can be provided
with its own ATG start codon and framed by suitable
restriction sites can be utilized. In addition, where
the intermediate vector which contains the promoter
sequence also contains, operably linked to the promoter,
20 codons which include an ATG start signal, any sequence
which can be placed in reading frame with the ATG can be
expessed as a fusion protein. For example, a portion of
the β-galactosidase coding sequence has been so ligated.
Thus, a panoply of carrier expression vectors for many
25 desired gene sequences can be constructed from an
intermediate carrier vector.

B.2   Expression Carrier Vectors for
Direct Transformation
The initial single cassette carrier expression
30 vectors which result from insertion of coding sequences
into the intermediate carrier vector are further
characterized in that the expression cassettes contained
in them are interchangeable and c mbinations of these can

-15-

be used to form carrier expression vectors which have
two, three or any desired number of cassettes. Thus, it
is possible to construct, for example, in a
straightforward fashion, a vector which contains not only
5 a cassette for a desired gene sequence, but also a
cassette for the expression of a selectable marker which
permits selection of the successfully transformed cells.
        ∗ The critical portions of the expression carrier
vector comprise one or more expression cassettes, each of
10 which contains a desired coding sequence operably linked
both to a promoter normally operable in plant cells, and
to a polyadenylation signal operable in plant cells. In
a preferred embodiment, at least one of the desired
coding sequences encodes a protein which results in a
15 phenotypic characteristic enabling selection of
transformed plant cells--i.e., a dominant selectable
marker. Also preferred are those embodiments wherein a
cassette-unique restriction site is located at one end of
the expression cassette in a single expression cassette
20 vector, and at the terminus of a series of expression
cassettes or at the junction between two cassettes in a
multiple cassette vector, and is, in this case, unique
not only to the particular expression cassette of its
location, but to the entire series or "DNA sequence
25 cassette". In instances where the expression carrier
vector is to be used as a donor vector for supplying an
additional expression cassette to a recipient, it is
further preferred that there be a second cassette-unique
restriction site at the expression cassette's other
30 terminus.
        In a preferred method of construction of
expression carrier vectors which contain more than one
expression cassette, two single expression cassette
vectors are used, one to provide simply an expression

-16-

cassette (a don r) and the other t pr vide its own
expression cassette as well as the vector sequences (the
recipient). In this method, the recipient vector is
cleaved with one restriction enzyme which cuts at a
5 cassette-unique site at the terminus of its expression
cassette; the donor expression cassette is excised by
treating the donor plasmid with two restriction enzymes,
one for each cassette-unique restriction site at the ends
of the expression cassette. The excised expression
10 cassette and the recipient plasmid are then ligated using
standard conditions to yield the resulting multiple
expression cassette vector.

         If the cassette-unique restriction sites are
chosen so that they yield compatible sticky ends (for
15 example, XhoI and SalI), the inserted expression cassette
can be oriented in either of two orientations, and the
position of the regenerated cassette-unique restriction
site will depend on which orientation has resulted. For
example, in the examples below, the expression cassettes
20 are bounded by XhoI and SalI restriction sites at the 5'
and the 3' termini, respectively. The recipient vector
is treated with SalI to form linear DNA with SalI sticky
ends. The excised donor expression cassette bears XhoI
and SalI sticky ends. Under these circumstances, if the
25 expression cassette is oriented so that the DNA sequence
is in the same direction as the recipient, a SalI site
will be regenerated at the terminus of the resulting DNA
sequence double cassette; if it is oriented in the
opposite direction, the cassette-unique SalI site will be
30 located between the two expression cassettes. Both
orientations will be functional, and both will provide a
suitable cassette-unique restriction site to permit the
double expression cassette vector to serve as a recipient
for the insertion of additional expression cassettes.

-17-

In summary, an intermediate carrier vector is
constructed and is part of the invention. It has,
reading in the 5' to 3' direction of the sense strand, a
first restriction site, a promoter normally operable in
5 plants, a polylinker, a polyadenylation signal operable
in plants, and a second restriction site. The polylinker
permits convenient insertion of a desired coding sequence
to generate a single cassette expression carrier vector
wherein the single cassette is bounded by the two
10 restriction sites. Mutiple cassette expression carrier
vectors can be constructed by excising the expression
cassette of one such vector which has been cleaved at
these two restriction sites and inserting it into another
using just one of the restriction sites of the recipient
15 vector, a procedure which can be repeated any desired
number of times. In a particularly preferred embodiment,
this process is made more efficient by utilizing
combinations of restriction sites such that one of the
sites is not regenerated after the ligation of two
20 expression cassettes.
         The nature of remaining vector fragment
portions of the expression carrier vectors just
described, which are suitable for direct transformation
into plant tissues under appropriate conditions, is less
25 critical than that required for other transformation
techniques, i.e., they need not contain sequences which
would be required for transformation using the mediation
of an infecting bacterium. Thus, these vectors may or
may not contain T-DNA border sequences from
30 A. tumefaciens, and their origins of replication may be
adaptable to either a narrow or broad range of hosts. In
other words, the Agrobacterium-dependent carrier
expression vectors described in paragraph B.3 below can
be used as "ordinary" expression carrier v ctors in

-18-

direct transformation. All of the illustrative
paragraphs below describe expression carrier vectors
which are suitable for direct transformation. Examples
of vectors constructed in paragraph G. describe such
5 vectors which are appropriate for both direct
transformation and for Agrobacterium-mediated
transformation.

   B.3   Expression Carrier Vectors Useful
         in Agrobacterium-Mediated Transformation
10   The expression carrier vectors described above
can be modified in their vector segments so as to adapt
them for use in plant cell transformation mediated by
bacteria. If A. tumefaciens is to be used as the
infectious agent, the modified vector will have at least
15 one A. tumefaciens T-DNA border sequence disposed
proximate the terminus of the expression cassette or the
series thereof. Preferably the expression cassette or
series is framed by a pair of such border sequences. In
order to permit the vector to replicate in the
20 A. tumefaciens host, while still permitting the vector to
have been constructed in, for example, E. coli, the
vector segment contains a broad host range bacterial
origin of replication. While these modifications do not
impair the ability of the expression carrier vectors to
25 transform plant cells directly, they are necessary to
permit transformation using the offices of the
intermediate bacterium.
         In construction of the modified vectors, the
preferred pivotal vector is comprised of an expression
30 cassette containing a desired coding sequence (preferably
that encoding a dominant selectable marker) operably
linked both to a promoter normally operable in plant
cells and a polyadenylation sequence operable in plant

-19-

cells framed by the right border (RB) and left border
(LB) sequences associated with A. tumefaciens. A
cassette-unique restriction site is disposed between one
of the border sequences and the terminus of the

5 expression cassette. The vector segment contains a broad
host range bacterial origin of replication.
             This basic expression carrier Agrobacterium-
adapted vector is exemplified below by pCMC91. In
pCMC91, the coding sequence encodes the dominant

10 selectable marker, a modifed APH-II, conferring
resistance against G418 and kanamycin. Derivatives of
the basic construction contain additional expression
cassettes so that these derivatives are useful in
expressing the coding sequence of any desired foreign

15 gene in the transformed plant cell, and, further, still
retain the selectable marker feature of the original
cassette. These expression cassettes can be conveniently
derived from the vectors already described above in
paragraph B.2. They are excised by use of the two

20 cassette-unique restriction sites at either end of the
cassette, and ligation of the cassette at the cassette-
unique restriction site between the border sequence and
the terminus of the APH-II cassette in the recipient
vector.

25   B.4   Methods of Transforming Plant Cells
                   Using Vectors of the Invention

             B.4.a.   Direct Transformation
             All of the expression carrier vectors of
the invention can be used to transform plant cells

30 directly. In this method, no bacterial infection is
employed. Rather, plant cells are treated under suitable
conditions directly with naked DNA, i.e., simply with a

-20-

suspension of the appropriate vector. At present, one
workable method to effect this transformation is to
suspend plant cell protoplasts with the plasmid DNA in
the presence of a suitable facilitator such as, for
5 example, polyethylene glycol (PEG). This technique is
described in greater detail in paragraph H. below.
Another method, which has been used less frequently,
employs microinjection into individual plant cells. This
method is more tedious, but does not require the
10 preparation of protoplasts. In any event, the vectors of
the invention are suitable for any workable direct
transformation technique and it should be understood that
the invention is not limited to their use in the
foregoing specific methods.

15   B.4.b.   Bacterial Mediated
                                 Transformation
                 Those vectors which are described in
paragraph B.3 are suitable for use in bacterial mediated
transformation of plant cell hosts. Two variations of
20 such methods are commonly employed. Both share the same
initial step--transformation of a suitable bacterial host
harboring a Ti plasmid bearing the virulence coding
regions. The bacterial host, now containing both the
expression carrier vector and the virulence-containing
25 plasmid, is either co-cultivated with plant cells,
preferably protoplasts, in tissue culture, or injected
directly into wounded plant tissue. These alternatives
are described in greater detail below.
                 Whichever alternative is used, the
30 infecting or co-cultivated Agrobacterium will contain
both the expression carrier vector and a plasmid bearing
the virulence region normally associated with a tumor-
inducing plasmid. It is preferred, in order to prevent

-21-

the resulting transformed cells from becoming tumorous,
that the virulence region reside on a plasmid lacking the
tumor-inducing functions normally carried in its T-DNA
region. Such a disarmed virulence region containing
5 plasmid may already be present in the host bacterial cell
as in the example hereinbelow, or, perhaps less
conveniently, a bacterial host devoid of tumor-inducing
plasmids may be transformed both with the carrier
expression vector and a disarmed virulence-encoding
10 vector, either simultaneously or sequentially. In a
preferred embodiment, an A. tumefaciens host already
containing a disarmed Ti plasmid is used as the recipient
for the expression carrier vector.

        To summarize, four illustrative methods of
15 transformation are:

        (1) The carrier expression vector is suspended
under suitable conditions with plant cell protoplasts to
permit direct transformation.

        (2) Microinjection.

20   If methods (1) or (2) are used, it is
immaterial whether the replicon is of narrow or broad
host range, and whether border sequences are present.
The vectors of both B.2 and B.3 may be employed.

        (3) The carrier expression vector is first
25 transformed or conjugated into Agrobacterium containing a
disarmed Ti vector and then the Agrobacterium co-
cultivated with plant protoplasts in tissue culture.

        (4) The carrier expression vector is first
transformed into Agrobacterium as above and the
30 Agrobacterium applied directly into wounded plant tissue.

        Methods (3) and (4) are suitable only for the
vectors of B.3.

-22-

C. Methods Employed for Vector Construction

Construction of suitable vectors containing the
desired coding and control sequences employs standard
ligation and restriction techniques which are well
5 understood in the art. Isolated plasmids, DNA sequences,
or synthesized oligonucleotides are cleaved, tailored, and
religated in the form desired.

Site specific DNA cleavage is performed by
treating with the suitable restriction enzyme (or
10 enzymes) under conditions which are generally understood
in the art, and the particulars of which are specified by
the manufacturer of these commercially available
restriction enzymes. See, e.g, New England Biolabs,
Product Catalog. In general, about 1 µg of plasmid or
15 DNA sequence is cleaved by one unit of enzyme in about
20 µl of buffer solution; in the examples herein,
typically, an excess of restriction enzyme is used to
insure complete digestion of the DNA substrate. Partial
digestions are occasionally specified. Incubation times
20 of about one hour to two hours at about 37°C are
workable, although variations can be tolerated. After
each incubation, protein is removed by extraction with
phenol/chloroform; this step may be followed by ether
extraction and the nucleic acid recovered from aqueous
25 fractions by precipitation with ethanol followed by
running over a Sephadex G-50 spin column. If desired,
size separation of the cleaved fragments may be performed
by polyacrylamide gel or agarose gel electrophoresis
using standard techniques. A general description of size
30 separations is found in Methods in Enzymology (1980)
65:499-560.

Restriction cleaved fragments may be blunt
ended by treating with the large fragment of E. coli DNA
polymerase I (Klenow) in the presence of the four

-23-

nucleotide triphosphates (dNTPs) using incubation times
of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6,
50 mM NaCl, 6 mM MgCl$_2$, 6 mM DTT and 0.1 mM dNTPs. The
Klenow fragment fills in at 5' sticky ends but chews back
5   single strands, even though the four dNTPs are present,
at 3' sticky ends. If desired, selective repair can be
performed by supplying only one of the, or selected,
dNTPs within the limitations dictated by the nature of
the sticky ends. After treatment with Klenow, the
10 mixture is extracted with phenol/chloroform and ethanol
precipitated followed by running over a Sephadex G-50
spin column. Treatment under appropriate conditions with
S1 nuclease results in hydrolysis of any single-stranded
portion.
15   Synthetic oligonucleotides are prepared by the
triester method of Matteucci, et al; (J Am Chem Soc (1981)
103:3185-3191). Kinasing of single strands prior to
annealing or for labeling is achieved using an excess,
e.g., approximately 10 units of polynucleotide kinase to
20 1 nmole substrate in the presence of 50 mM Tris, pH 7.6,
10 mM MgCl$_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles
γ^{32} P ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM
EDTA.
        Ligations are performed in 15-30 1 volumes
25 under the following standard conditions and temperatures:
20 mM Tris-Cl pH 7.5, 10 mM MgCl$_2$, 10 mM DTT,
33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP,
0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky
end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA
30 ligase at 14°C (for "blunt end" ligation).
Intermolecular "sticky end" ligations are usually
performed at 33-100 µg/ml total DNA concentrations
(5-100 nM total end concentration). Intermolecular blunt
end ligations (usually employing a 10-30 fold molar

-24-

excess of linkers) are performed at 1 μM total ends
concentration.

In vector construction employing "vector
fragments," the vector fragment is commonly treated with
5 bacterial alkaline phosphatase (BAP) in order to remove
the 5' phosphate and prevent religation of the vector.
BAP digestions are conducted at pH 8 in approximately 150
mM Tris, in the presence of Na+ and Mg+2 using about 1
unit of BAP per μg of vector at 60° for about one hour. In
10 order to recover the nucleic acid fragments, the
preparation is extracted with phenol/chloroform and
ethanol precipitated and desalted by application to a
Sephadex G-50 spin column. Alternatively, religation can
be prevented in vectors which have been double digested
15 by additional restriction of the unwanted fragments.

In the constructions set forth below, correct
ligations for plasmid construction are confirmed by
transforming E. coli strain MM294 (Talmadge, K., et al,
Gene (1980) 12:235; Meselson, M., et al, Nature (1968)
20 217:1110) obtained from E. coli Genetic Stock Center,
CGSC #6135, or other suitable host with the ligation
mixture. Successful transformants are selected by
ampicillin, tetracycline or other antibiotic resistance
or using other markers depending on the mode of plasmid
25 construction, as is understood in the art. Plasmids from
the transformants are then prepared according to the
method of Clewell, D. B., et al, Proc Natl Acad Sci
(1969) 62:1159, following chloramphenicol amplification
when feasible (Clewell, D. B., J Bacteriol (1972)
30 110:667) and analyzed by restriction and/or sequenced by
the dideoxy method of Sanger, F., et al, Proc Natl Acad
Sci (USA) (1977) 74:5463, as further described in
Messing, et al, Nucleic Acids Res (1981) 9:309, or by the

-25-

method of Maxam, et al, _Methods in Enzymology_ (1980)
_65_:499.

        The following examples illustrate the
construction of suitable intermediate carrier vectors,
5 single cassette and double cassette expression carrier
vectors, and bacterial adapted derivatives thereof
suitable for the transformation techniques described.
In these vectors, the nopaline synthase (NOS) promoter
of pTiT37 is utilized along with the polyadenylation
10 signal for this gene. Of course, other promoter
sequences normally operable in plant cells can be used as
well as other polyadenylation signals operable in plants.
Suitable alternate promoters include the maize promoter
for alcohol dehydrogenase-1 or alchohol dehydrogenase-2
15 (Gerlach, W. L., et al, _Proc Natl Acad Sci (USA)_ (1982)
_79_:2981), cauliflower mosaic virus promoters (Daubert,
S., et al _Virology_ (1982) _122_:444), and wheat promoter
associated with the small subunit of ribulose biphosphate
carboxylase (Broglie, R., et al, _Biotechnology_ (1983)
20 _1_:55). The foregoing are readily available in the art,
but, of course, other suitable promoters can be used.
Other polyadenylation signals which are available
presently include those found on any of the above genes,
or those of Schuler, et al (Schuler, M. S., et al,
25 _Nucleic Acid Res_ (1982) _10_:8225). The coding sequences
inserted below also are not limiting. Any available
coding sequence could be substituted, either with an ATG
start codon so as to result in an mature protein, or in
reading frame with a portion of the NOS sequence.

30 D. Construction of the Intermediate Carrier
        Vectors pCMC60 (and pCMC59)
        The construction of the intermediate carrier
vector pCMC60 is outlined in Figure 1. This plasmid is

-26-

constructed by manipulating the nopaline synthase (NOS)
gene derived from the 3.2 kilobase (kb) HindIII-23 DNA
fragment in the T-DNA region of A. tumefaciens Ti plasmid
pTiT37 (Bevan, M., et al, Nucleic Acid Res (1983) 11:369;
5 Depicker, A., et al, J Mol Appl Genet (1982) 1:5613).
The HindIII-23 fragment, containing the complete NOS
gene, obtained from pTiT37, was cloned into the HindIII
site of pBR325 (Bolivar, F., et al, Gene (1978) 4:121) to
obtain the starting plasmid pCMC1 (8.9 kb). pCMC1 served
10 as the source of the NOS promoter, polyadenylation
signal, and in the bacterial adapted plasmid derivatives,
the "right" T-DNA border sequences.

          The construction of pCMC60 employs a number of
intermediate plasmids as shown in Figure 1 and below.
15 These are: pCMC30 which provides the NOS promoter
modified to contain a HindIII site at its 3' terminus,
pCMC39 which provides the NOS promoter modified to
contain a XhoI site at its 5' terminus, and pCMC49 which
provides the polylinker, the polyadenylation signal and a
20 bacterial host replicon.

          D.1   pCMC30
          pCMC30 was constructed to contain the NOS
promoter modified to contain a HindIII site at the 3'
terminus. A primer repair reaction, essentially as
25 outlined by Messing, J., Third Cleveland Symposium on
Macromolecules: Recombinant DNA (1981). A. Wiseton, ed.,
Elsevier Amsterdam, pp.143-153; was employed to effect
this modification. This was done as follows: pCMC1 was
digested with Sau3AI to excise a 346 bp fragment
30 containing the NOS promoter. This fragment was isolated
and cloned into the BamHI site of the single stranded M13
bacteriophage mp10RF (Messing, J., et al, Gene (1982)
19:269) to produce mCMC10, a 7.57 kb bacteriophage,

-27-

whose construction was confirmed by digestion with SstII
and HindIII to release the expected 260 bp fragment.

The sequence of the Sau3AI insert was confirmed
to comprise the NOS promoter and the sequences
5 surrounding the 3' terminus of the promoter were
confirmed by dideoxy sequencing to conform to the
expected sequence as disclosed by Depicker, A., et al,
J Mol Appl Genet (1982), Vol. 1, p. 566. The 3' terminus
of the promoter was modified using a primer repair
10 reaction by annealing with the synthetic pentadecamer
5'-pTTGCAGATTATTTGG-OH 3' which complements this fragment
so as to include the first A of the ATG codon and 14
upstream nucleotides. In the primer repair, 50 µg of
single-stranded mCMC10 DNA was digested with HaeIII and
15 the 597 bp fragment isolated. One µg of the isolated
fragment was annealed with the pentadecamer (supra) and
extended with T4 DNA polymerase I in the presence of
dNTPs under standard conditions. The remaining single-
stranded region at the 3' end was simultaneously degraded
20 to the 5' terminus of the annealed primer. The resulting
primer-repaired double-stranded fragment thus terminates
at the 3' end after the A of the ATG codon at position 1
of the NOS coding sequence. This double-stranded
fragment was digested with EcoRI and the 320 bp EcoRI/
25 blunt DNA fragment purified and cloned into pUC13 (a
freely available pBR322 derivative containing
polylinkers; see Messing, J., et al, Gene (1982) 19:269)
as follows:

pUC13 (10 µg) was digested with HindIII and
30 then treated with DNA polymerase I (Klenow fragment) in
the presence of dNTPs and then digested with EcoRI. The
vector fragment was ligated with the 320 bp EcoRI/blunt
promoter fragment above and the ligation mixture
transformed into E. coli MM294. Successful transformants

-28-

were selected by Amp^R, and the required plasmid, pCMC30,
confirmed by restriction analysis with SstII/HindIII to
yield the expected 188 bp DNA fragment.

5   The correct construction was further confirmed
by dideoxy sequencing to contain a recreated HindIII site
at the junction of the insert with pUC13; the plasmid
also provides EcoRI, SstI, and SmaI/XmaI sites 5' to the
promoter sequences. Thus, pCMC30 provides the NOS
promoter as a convenient EcoRI, SstI, or SmaI (XmaI)/
10 HindIII cassette.

D.2   pCMC39
pCMC39 is another intermediate plasmid and it
contains the NOS promoter modified at the 5' terminus to
contain a XhoI site. The XhoI site was excised from
15 pBW14 (4375 bp), which is a pBR322 derivative into which
a XhoI-containing octanucleotide (5'-CCTCGAGG-3') was
ligated into a Klenow repaired, HindIII digest of pBR322.
pBW14 was digested with XhoI, treated with DNA polymerase
I (Klenow fragment) in the presence of dNTPs and then
20 treated with EcoRI. The approximately 40 bp EcoRI/XhoI
(blunt) fragment was isolated, and ligated in a two-stage
ligation to a SmaI/EcoRI digest of pCMC29. pCMC29
contains an unmodified NOS promoter whih was originally
obtained from pCMC1 by digestion of pCMC1 with Sau3AI and
25 inserted into the BamHI site of pUC13. The ligation
mixture was used to transform E. coli MM294 and Amp^R
colonies were selected. Successful transformants were
screened for the desired 3.1 kb plasmid, designated
pCMC39.

30   pCMC39, therefore, contains the NOS promoter
modified at the 5' terminus to contain a XhoI site
immediately upstream as shown in Figure 1. Its
construction was confirmed by restriction analysis.

-29-

D.3 pCMC49

pCMC49 was constructed to contain the NOS
polyadenylation signal and a polylinker segment through
the intermediate plasmid pCMC20, which, in turn, was
5 constructed using a digest of pCMC1 as the source of the
relevant sequences, combined with rsal03 used as a source
of the polylinker for the expression plasmid. pCMC1 was
digested with Sau3AI and the 250 bp fragment containing
the polyadenylation signal from the NOS gene isolated.
10 The isolated fragment was ligated with BamHI digested
pUC8 (a freely available pBR322 derivative containing a
polylinker for convenient constructions; see Messing, J.,
et al (supra)) and the ligation mixture was transformed
into E. coli MM294. Successful (AmpR) transformants were
15 screened for the expected 3.0 kb plasmid and construction
was confirmed by restriction analysis. pCMC20 contains
unique SstI and SmaI (XmaI) sites 5' to the
polyadenylation signal and SalI, PstI, HaeII, HindIII and
BssHII sites 3' to the signal.
20   To give pCMC49, pCMC20 was digested with XmaI,
blunt-ended with Klenow fragment and the four dNTPs, and
then digested with SalI. The 260 bp desired terminator
signal fragment was purified and ligated to BamHI(Klenow
repaired)/SalI digested rsal03 purified vector-plus-
25 polylinker fragment and the ligation mixture transformed
into E. coli MM294. AmpR transformants were selected and
screened for the desired plasmid, pCMC49, which contains
the signal fragment framed by a 5' polylinker containing
HindIII, XbaI, BglII, PstI, BamHI and XmaI/(SmaI) sites,
30 and a 3' SalI site as well as the origin of replication
from rsal03. (rsal03 is a pBR322 derivative in which (i)
a polylinker containing HindIII XbaI, BglII, PstI, BamHI,
and SalI sites is substituted for the 621 bp HindIII/SalI
vector fragment in the tetracycline resistance region, by

-30-

insertion of the small HindIII-BamHI fragment from VX
(Seed, B., Nucleic Acid Res (1983) 11:2427) into the
large HindIII-BamHI fragment of pBR322, and (ii) the
514 bp RsaI DNA fragment from bacteriophage M13RF
5 containing the origin of replication is adapted with
HindIII linkers, cut with HindIII and inserted at the
HindIII site. In rsal03, the M13RF origin of replication
is oriented in the same direction as the bla gene on the
pBR322 portion.)

10   D.4 Completion of pCMC60
           The 3' unmodified, XhoI-5' supplemented, NOS
promoter from pCMC39 and the polylinker-signal sequences
along with the rsal03 vector fragment from pCMC49 were
ligated to give pCMC59, the penultimate plasmid in the
15 preparation of pCMC60, which is also a (modified)
intermediate carrier vector. To do this, pCMC39 was
digested with EcoRI and HindIII, the digest ligated to
EcoRI/HindIII digested pCMC49 and the ligation mixture
used to transform E. coli MM294. Amp^R colonies were
20 screened for the presence of a 4.48 kb plasmid containing
unique EcoRI and HindIII sites as well as the remaining
expected restriction sites.
           The selected plasmid, pCMC59, contains the
3' unmodifed NOS promoter preceded by several 5'
25 restriction sites which is upstream from the polylinker
and the polyadenylation signals followed at the 3'
terminus by additional restriction sites as well as the
vector fragment of rsal03, which includes the Amp^R gene
and replicon. pCMC59 is an alternate form of
30 intermediate carrier vector which permits the insertion
of a desired coding sequence so as to yield a fusion
protein with the first approximately 15 amino acids of

-31-

the NOS sequence provided the sequence is inserted in
appropriate reading frame.

The more convenient intermediate carrier
vector, pCMC60, which permits the insertion of a coding
5 sequence for a mature protein in front of the HindIII
site provided by the modified promoter was constructed
employing pCMC59 and pCMC30. pCMC30 containing the
3'-modified NOS promoter was digested with SstII and
HindIII and the 188 bp promoter-containing fragment
10 isolated. pCMC59 was also digested with SstII and
HindIII and the vector fragment purified. A ligation
mixture of these two purified fragments was transformed
into E. coli MM294 and selected on medium, containing
100 µg per ml ampicillin and 40 µg per ml X-gal, for
15 white, Amp^R colonies. (Colonies which are Amp^R due to
undesired pCMC30 transformation are blue; pCMC30 contains
the lac operator which results in depression of
β-galactosidase in MM294, thus giving blue colonies on
X-gal plates, if present). White Amp^R colonies were
20 screened for transformants containing the desired
construction, pCMC60.

Thus, pCMC60 contains a narrow host range
bacterial host vector segment from rsal03 and a gene
control sequence cassette which, in a 5' to 3' direction,
25 contains a cassette-unique restriction site (a XhoI site,
among others), the NOS promoter modified at its 3' end to
contain a HindIII site immediately preceding what would
be the ATG initiation codon in the Ti plasmid nopaline
synthase gene, a polylinker segment, and a
30 polyadenylation signal derived from the NOS gene
followed, finally, by a second cassette-unique
restriction site, SalI.

-32-

E. Construction of Single Cassette
   Expression Carrier Vectors

Single cassette expression carrier vectors were
constructed using pCMC60 as the intermediate carrier
5 vector for the control and replication sequences as
follows:

pCMC70 contains coding sequences for a
truncated and modified APH-I protein (herein designated
mtAPH-I) encoding an aminoglycoside phosphotransferase
10 activity derived from the transposon Tn601 (also known as
Tn903) (Sharp, P. A., et al, J Mol Biol (1973) 75:235;
Oka, A., et al, J Mol Biol (1981) 147:217). The protein
encoded by this sequence is capable of conferring
resistance to the antibiotics G418 or kanamycin.

15   pCMC71 contains the coding sequence for APH-II,
an enzyme conferring similar antibiotic resistance, which
is encoded on the transposon Tn5 (Jorgensen, R. A., et
al, Mol Gen Genet (1979) 177:65).

Also constructed were:
20   pCMC102 wherein the sequence insert encodes
β-interferon.

pCMC103 wherein the insert encodes
chloramphenicol acetyltransferase (CAT) which confers
resistance to chloramphenicol.

25   pCMC121 which encodes Bt-toxin, an endotoxin
from Bacillus thuringiensis strain HD-1, which is lethal
to the larvae of certain insects.

The foregoing are intended, of course, as
merely exemplary. The following paragraphs describe the
30 origins of the various coding sequences and their
insertion into pCMC60.

-33-

E.1 pCMC70

pDG144 contains the modified coding sequence
for a truncated, modified APH-I protein (mtAPH-I). The
sequence had been modified by destroying the Xma (SmaI)
5 site at the codons for amino acids 93/94 by converting
the CCC codon encoding proline (93) to the also proline
encoding CCT, and by destroying the HindIII recognition
site at amino acids 175/176 by converting the lysine
encoding AAG codon to the also lysine encoding AAA. In
10 addition, the N-terminal sequence was truncated and
placed immediately downstream from a HindIII recognition
site by altering the wild type sequence immediately
before the ATG start codon from GGTGTT to AAGCTT,
deleting the codons for amino acids 2-10 and fusing the
15 ATG start codon to the codon for amino acid 11.
Therefore, the N-terminal sequence immediately preceded
by HindIII recognition site now reads AAGCTTATGTCGAGG.

pDG144 was digested with HindIII and PvuII to
excise a 1.21 kb fragment terminating in the 3'
20 untranslated sequence of mtAPH-I, and ligated with
HindIII and SmaI digested pCMC60 in a two-phase ligation
under sticky end and then blunt end conditions. The
ligation mixture was then used to transform E. coli MM294
and Amp^R colonies selected and screened for the presence
25 of the desired 5.6 kb recombinant plasmid, pCMC70. The
correct construction was confirmed by restriction
analysis.

E.2.a. pCMC71

pCMC71 encoding APH-II was constructed
30 similarly. The APH-II gene was obtained by digestion of
pAM1 (de Prammond, A., et al, Biotechnology (1983) 1:262-
269), a plasmid which confers kanamycin resistance, with
BglII and SmaI to yield a 1.0 kb DNA fragment containing

-34-

the desired APH-II coding sequence with an additional 36
bp preceding the translation initiation codon. The
polylinker sequence in pCMC60 contains BglII and SmaI
recognition sites in the correct orientation to accept
5 and express the resulting APH-II fragment, and thus this
fragment was ligated with a BglII/SmaI digest of pCMC60,
the mixture digested with BamHI to inactivate unwanted
fragments and used to transform E. coli MM294. Amp^R and
chloramphenicol sensitive colonies were screened for the
10 presence of a 5.35 kb plasmid. pCMC71 was analyzed by
restriction analysis and shown to have lost the SmaI
site; the remainder of the structure was as expected.

E.2.b. Construction of pCMC72

pCMC72 was constructed from pCMC71 as
15 shown in Figure 5. This construction removes an out of
frame ATG codon in the 5'-noncoding region of the APH-
II mRNA, and thus results in an elevated level of APH-II
protein production relative to pCMC71.

pCMC71 was partially digested with PstI to
20 open a PstI site located within the APH-II coding region,
177 bp from the amino terminal ATG. The plasmid was
then digested to completion with BglII, and both the 207
bp BglII/PstI fragment containing the N-terminal coding
sequences and a portion of the 5' untranslated region,
25 and the almost full-length vector fragment (missing this
207 bp fragment) were isolated. The 207 bp fragment was
then digested with Sau3AI, which removed an additional 24
nucleotides from the BglII end but left a four nucleotide
sticky end (GATC) which is compatible with the BglII
30 sticky end. The resulting Sau3AI/PstI fragment was then
ligated with the isolated BglII/PstI vector fragment of
pCMC71, and transformed into E. coli to Amp^R. The

-35-

correct plasmid, pCMC72, was confirmed by restriction
analysis.

pCMC72 is identical to pCMC71 except for a
24 bp deletion at the 5' end of APH-II which removes an
5 out of frame ATG codon and the bacterial ribosome binding
site.

E.3 · pCMC102

pCMC102 contains the β-interferon gene in
operable linkage to the components of the carrier
10 cassette. pβltrp3-4-1, a plasmid containing the β-IFN
coding sequence under the control of the trp promoter,
was used as a source of the β-interferon gene. It was
digested with HindIII and XhoII and the 502 bp fragment
containing the mature β-interferon coding sequence was
15 isolated. pCMC60 was digested with HindIII and BglII and
the fragments mixed with the purified β-interferon
fragment, ligated, and then treated with XbaI to
eliminate undesired ligation products. The ligation
mixture was used to transform E. coli MM294 and selection
20 made for AmpR. Successful transformants were screened
for the desired 4.89 kb plasmid and the correct
construction, pCMC102, was confirmed by restriction
analysis.

E.4 pCMC103

25 The gene encoding chloramphenicol acetyl
transferase (CAT) which encodes a protein conferring
chloramphenicol resistance was excised from pBR325
(Bolivar, F., et al, Gene (1978) 4:121) by isolating a
775 bp fragment generated by digestion with TaqI. The
30 TaqI fragment was subcloned into the AccI site of the
polylinker in pUC13 to give an intermediate plasmid which
contains the coding sequence for CAT between a HindIII

-36-

site 45 bp upstream of the ATG start codon and an XmaI
site 100 bp 3' of the TAA termination codon. The
HindIII/XmaI fragment was excised from the intermediate
plasmid and purified. pCMC60 was digested with XmaI and
5 HindIII and the linear DNA ligated with the foregoing
fragment, followed by BglII digestion to inactivate
undesired products. The ligation mixture was transformed
into E. coli MM294 and successful colonies were selected
for Amp^R. The desired vector, pCMC103, was confirmed by
10 restriction analysis.

        E.5 pCMC121
        pCMC121, which is a one cassette carrier
expression vector for Bt-toxin, was constructed using
pCMC60 in a manner analogous to the foregoing
15 constructions. Its construction is outlined in Figure 2.
        The recombinant plasmid, pES1 (Wong, et al,
J Biol Chem (1983) 258:1960 and Schnepf and Whiteley,
Proc Natl Acad Sci (USA) (1981) 78:2893), is a derivative
of pBR322 containing as an insert the coding sequences
20 for the Bt-toxin. See also European Application
Publication No. 0063949 (November 3, 1982). pES1, in
E. coli K12/HB101 is deposited under the terms of the
Budapest Treaty at the ATCC under No. 31995. pES1 was
mutagenized with the bacterial transposon, Tn5 according
25 to the method of Guyer, M. S., et al, Methods Enzymol
(1983) 101:362 to insert a XhoI recognition sequence 5'
to the Bt-toxin. A 5.8 kb XhoI fragment was isolated
from the pES1-Tn5 plasmid and inserted into the SalI site
of pUC9. The ligation mixture was used to transform
30 E. coli K12 JM83 (available from BRL) and the successful
transformants selected by Amp^R and lac-. Successful
transformants were screened for the presence of the

-37-

desired 8.6 kb plasmid, and the construction of the
desired pSYC823 confirmed by restriction analysis.
          pSYC823 was further modified to place the
appropriate restriction sites at the termini of the
5 coding sequences. To create a HindIII site at the 5'
terminus, pSYC823 was digested to completion with NdeI,
and blunt ended with Klenow in the presence of dATP and
dTTP. After complete digestion with HindIII, the DNA was
ligated into the phage vector M13mp8 which had been
10 digested with SmaI and HindIII. The desired phage
(Intermediate I) containing the 1.8 kb NdeI to HindIII
amino-terminal region of the Bt-toxin was identified by
miniprep DNA analysis (Holmes, D. S., et al, Anal Biochem
(1981) 114:193). The single-stranded DNA form of the
15 desired phage was modified to obtain a HindIII site just
5' of the ATG initiation codon by primer directed
mutagenesis, as described by Moller, M. P., et al,
Nucleic Acids Res (1982) 10:6487, using the primer

          5' GAGGTAA CTTATGG 3' (Bt-toxin sequence)
20   3' CTCCATTCGAATACC 5' (primer)
                  HindIII

which contains a 1 base insertion mismatch for the
sequence 5' of the ATG start codon and a HindIII site as
shown. The resulting modified vector was designated
25 mp8BtRF.
          A 3' HindIII site was placed adjacent the
Bt-toxin coding region in pSYC823 as follows: Another
sample of pSYC823 was partially digested with NdeI,
completely digested with PstI, and blunt ended using
30 Klenow in the presence of all four dNTPs. The 7.0 kb
fragment was purified, self-ligated, and then transformed
into E. coli MM294. The desired intermediate plasmid is

-38-

designated pCMC120 and represents pSYC823 with a 1.6 kb
deletion of <u>Bacillus</u> sequences from the unique PstI site
of the vector to the NdeI site at the 3' end of the
Bt-toxin coding region. This deletion placed the HindIII
5 site of the pUC9 vector directly adjacent the 3' end of
the toxin coding region and destroyed the PstI site.
        The Bt-toxin coding sequence was inserted into
the pCMC60 intermediate vector in two portions, the 5'
portion from the mp8BtRF 5' HindIII modified segment, and
10 the 3' portion from the pCMC120 3' HindIII modified
Bt-toxin coding sequence of the intermediate plasmid
above.
        mp8BtRF was digested to completion with HindIII
and SstI and the 1.4 kb fragment isolated. The second
15 fragment was obtained by digesting pCMC120 to completion
with SstI, then partially with HindIII and purifying the
2.3 kb DNA fragment from the HindIII site of the pUC9
vector portion to the SstI site located within the gene.
The two foregoing fragments were ligated with HindIII
20 digested, BAPed pCMC60, and transformed into <u>E. coli</u>
MM294. Amp^R colonies were selected and screened for the
presence of pCMC121, which contains the entire coding
sequence for Bt-toxin under the control of the NOS
promoter and polyadenylation signal sequences.

25   E.6 <u>Construction of an Intermediate</u>
                 <u>and Expression Carrier Plasmid Suitable</u>
                 <u>for Expression of a Fusion Protein</u>
        pCMC101 is analogous to pCMC60 except that,
instead of the NOS promoter which terminates in a
30 convenient HindIII site suitable for the insertion of a
mature coding sequence, pCMC101 has a chimaeric segment
with the NOS promoter fused to coding sequences for
β-galactosidase. Subsequent insertion of desired coding

-39-

sequence in reading frame with the β-galactosidase codons
results in a fusion protein containing both
β-galactosidase sequences and amino acid sequences
corresponding to the inserted codons.   It is also an
5 expression carrier vector in that it is capable of
effective expression of the NOS-β-galactosidase fusion.
The construction is outlined in Figure 3.
        Plasmid pMC1403 (Casadaban, M. J., et al,
J Bacteriol (1980) 143:971) contains the
10 β-galactosidase coding sequence with a polylinker at the
5' end.   To provide a convenient 3' terminal restriction
site, the lac-Z portion of pMC1403 was excised by
digestion with EcoRI (which cleaves in the polylinker) and
ClaI which cleaves just 3' of the lac-Z gene (Buchel, D.
15 E., et al, Nature (1980) 283:541).   The EcoRI/ClaI 3.4
kb fragment was cloned into EcoRI/ClaI digested pOG2326,
a bifunctional replicon capable of replication in both
E. coli and B. subtilis and derived from pBR322, to
obtain pDH5425.   pDH5425 was digested with BamHI to
20 obtain a 3.8 kb fragment spanning the BamHI site of the
polylinker sequence at the lac-Z 11th codon to the BamHI
of the Tet^R gene of the vector plasmid.   The BamHI
fragment was subcloned into the BamHI site of pUC13 to
give pCMC9163 which thus has a 5' terminal polylinker on
25 the lac-Z gene as follows:

        amino acid #:   9   10   11   12   13

                          ___ ___ ___ ___ ___
                      5' ACT CTA GAG GAT CCC-----lacZ-3'
                             XbaI   BamHI

        To construct the chimaeric promoter/coding
30 sequence, the pCMC29 intermediate plasmid (paragraph
D.2), which contains the unmodified NOS promoter, was

-40-

used. This plasmid has a polylinker region following the
codon 16 of the original NOS coding sequence as follows:

amino acid #: 16   17* 18* 19* 20* 21* 22* 23* 24* 25* 26*

5'- GAT CCT CTA GAG TCG ACC TGC AGC CCA AGC TTG
Sau3AI/BamHI XbaI   HindIII
            fusion

where * represents codons substituted for the native NOS
sequence.

The XbaI sites of pCMC29 and pCMC9163 are in
identical reading frames. Further, there is a unique
HindIII site at the 3.' end of the polylinker in pCMC29
and at the 3' end of the β-galactosidase coding sequences
in pCMC9163. Therefore, both plasmids were digested to
completion with HindIII and XbaI, the fragments ligated,
and the ligation mixture was transformed into E. coli
MM294. Successful colonies were selected for AmpR.

The resulting plasmid, pCMC17, thus comprises
the restriction sites EcoRI, SstI, SmaI(XmaI) of the
pUC13 polylinker, the NOS promoter and the amino terminal
coding region for NOS fused in correct reading frame to
the lac-Z gene, the coding sequences for β-galactosidase
downstream from codon 18, followed by a HindIII site.

To complete the construction of the
intermediate (and expression) carrier vector pCMC101
containing the chimaeric NOS/lac-Z sequences, pCMC60 was
digested with EcoRI and HindIII and the vector fragment
ligated with the 3.4 kb fragment obtained from pCMC17 by
digestion with EcoRI and HindIII containing the NOS
promoter and amino terminal codons and fused lac-Z gene.
The resulting vector, pCMC101, was obtained from
successful E. coli MM294 transformants selected for AmpR

-41-

by screening for the desired 7.9 kb plasmid and confirmed
by restriction analysis.

F.   Construction of Double Cassette
     Carrier Vectors

5   Because each of the expression carrier vectors
has convenient cassette-unique restriction sites, both 5'
and 3' of the expression cassette, these cassettes can be
recombined into multiple cassette vectors in a
straightforward manner. Since the coding sequence in
10 pCMC71 for APH-II results in a selectable marker
characteristic, pCMC71 was used as a recipient vector for
the expression cassettes of the other carrier expression
vectors described above. Of course, interchange could be
made between any of the various carrier vectors
.15 constructed.
            pCMC77 contains a double cassette which
includes the APH-II coding sequence and the β-interferon
sequence from pCMC102. To construct this vector, pCMC102
was digested to completion with XhoI and SalI, and the
20 desired 1.1 kb DNA fragment purified. The fragment was
ligated with SalI digested, BAPed pCMC71, and the
ligation mixture was transformed into E. coli MM294.
Amp^R colonies were screened for the presence of the
desired 6.5 kb plasmid, pCMC77. The correct construction
25 was confirmed by restriction analysis. As the ligation of
XhoI and SalI sticky ends does not regenerate either a
SalI or XhoI site, the resulting vector has XhoI and SalI
sites which remain "cassette-unique" at the 5' and 3'
termini of the series, if the donor expression cassette
30 is unidirectional with the recipient vector DNA
sequences; the SalI site is between the expression
cassettes if the opposite orientation results (see
paragraph B.2 above).

-42-

        Similar XhoI/SalI digestions were used to
prepare the CAT cassette from pCMC103, the mtAPH-I
cassette from pCMC70, and the Bt-toxin cassette from
pCMC121. These fragments were ligated into SalI digested
5 pCMC71 to form the double-cassette expression carrier
vectors below:

  pCMC78 containing the APH-II and CAT cassettes,
  pCMC79 containing the APH-II and mtAPH-I cassettes,
  pCMC75, containing the APH-II and Bt-toxin cassettes,

10   The NOS-lac-Z fusion cassette was obtained from
pCMC101, because there was no XhoI site 5' to the gene,
by digestion instead with EcoRI (a 5' cassette-unique
site), and SalI. This 5' EcoRI/3'SalI galactosidase
chimaera was ligated into pCMC71 which had been digested
15 with EcoRI and XhoI to give a double cassette recombinant
plasmid (pCMC76), selected following minipreps.

G. Construction of Single Cassette and Double Cassette
    Expression Carrier Vectors Adaptable to Agrobacterium
    Mediated Transformation
20   pCMC90, pCMC91 and pCMC92 are single cassette
expression vectors for the mtAPH-I or APH-II genes framed
by border sequences and having a broad host range
bacterial origin of replication. These constructions are
outlined in Figure 4. As shown in Figure 4, pCMC91 is
25 constructed from pCMC90 by replacing the mtAPH-I coding
region with the APH-II coding sequence from pCMC71.
pCMC92 is formed from pCMC90 using pCMC72 instead of
pCMC71. These plasmids are suitable for use in
Agrobacterium mediated transformations.

-43-

G.1   Construction of pCMC90

pCMC90 was constructed from the intermediate
vectors described below by inserting a 2.8 kb EcoRI/SalI
cassette from pCMC80, which contained the mtAPH-I coding
5   sequences with suitable control segments along with right
border (RB) T-DNA into a broad spectrum replicating host
plasmid bearing the left border (LB) regions, pCMC25.

G.1.a.   Construction of pCMC80

pCMC70, prepared as described in paragraph
10 E.1, is a carrier expression vector for the modified,
truncated (mt) dominant selectable marker, mtAPH-I. It
contains the NOS promoter as an EcoRI/HindIII cassette
immediately preceding the coding sequence. In pCMC80,
this cassette is replaced by a larger EcoRI/HindIII
15 cassette, which cassette contains the RB sequence from
pTiT37 along with the NOS promoter modified to have a
HindIII site at the 3' end of the promoter as in pCMC70
preceding the site for translation initiation as in
pCMC70.

20   pCMC1 was digested with HindIII, treated
with PolI Klenow fragment in the presence of dNTPs, and
then digested with SstII. The 1.1 kb HindIII(blunt)/
SstII fragment was isolated and ligated with DNA
fragments obtained by digesting pCMC70 with XhoI,
25 treating with Klenow and the dNTPs, and then digesting
with SstII. Approximately 200 ng of the DNA from the
ligation mixture was used to transform E. coli MM294 to
AmpR.

Successful colonies were screened for the
30 desired 6.55 kb recombinant plasmid pCMC80, which
contains the Ti T-DNA RB, the NOS promoter, the mtAPH-I
coding sequences, and the NOS polyadenylation signals
followed by a SalI site. Although the fusion of PolI

-44-

treated XhoI and HindIII sites would have been expected
to regenerate a HindIII site, the selected plasmid pCMC80
had lost this site. The foregoing sequences are
available as a 2.8 kb EcoRI/SalI DNA fragment, the 5'
5 XhoI site of pCMC70 having been lost. Thus, pCMC80
exemplifies a carrier expression vector for a foreign
gene with a narrow host range baterial origin of
replication, containing a right border sequence properly
placed with respect to the transcription cassette.

10   G.1.b. Construction of pCMC25
          pCMC25 was constructed as follows: The
LB sequence was obtained from pTiT37 by digesting with
EcoRI and isolating the 1.6 kb fragment 29 using standard
methods. This fragment was cloned into the EcoRI site of
15 pAM1, a derivative of pBR325 in which a 1.5 kb HindIII/
SalI DNA fragment from transposon Tn5 containing the
APH-II antibiotic resistance gene had been substituted
for the HindIII/SalI region of the Tet^R gene.
         The ligation mixture containing the thus
20 modified pAM1 vector was cloned into E. coli MM294 and
selected for Amp^R. Successful colonies were screened for
the desired 8.1 kb plasmid, pCMC5, which contained the
LB region of T-DNA contiguous with the APH-II gene.
         pCMC5 was further modified to provide a
25 broad spectrum bacterial origin of replication by
utilizing the vector portions of RSF1010 (Bagdasarian,
M., et al, Gene (1981) 16:237), a broad host range,
multicopy plasmid containing a sulfonamide resistance
(Su^R) gene and capable of replicating both in E. coli and
30 in A. tumefaciens. To do this, pCMC5 was partially
digested with BglII and then further digested with SalI
to produce the desired 3.4 kb BglII/SalI fragment
containing the T-DNA LB sequence and adjacent APH-II

-45-

gene. The fragment was repaired with Klenow and dNTPs to
provide a blunt-ended fragment having the sequence 5'-
GATCT-LB-APH-II-GTCGA-3'. This blunt-ended fragment was
ligated with SstI digested, Klenow repaired RSF1010 which
5 thus provided a vector sequence with 5' C and 3' G
terminal nucleotides. The ligation mixture was
transformed into E. coli MM294 and successful
transformants selected by KanR SuR and successful
transformants screened for the appropriate 12.7 kb
10 plasmid. Correct construction of pCMC15 was confirmed by
restriction analysis: the desired construction contains
a single SalI site at the point of ligation of the APH-II
DNA but has lost the SstI and BglII sites.

            pCMC15 was further modified to insert a
15 polylinker region adjacent the T-DNA-LB segment. The
polylinker was derived from pUC13 by digestion with
EcoRI, repaired with Klenow and all four dNTPs, followed
by further digestion with SalI. This provided a 32 bp
EcoRI(blunt)/SalI fragment containing SstI, SmaI(XmaI),
20 BamHI, XbaI, and SalI sites, which was isolated by
standard methods. This fragment was ligated with an
isolated 10.2 kb fragment produced by partial digestion
of pCMC15 with EcoRI to open the EcoRI site between the
LB and APH-II, repairing with Klenow, and further
25 digesting with SalI. The ligation mixture was trans-
formed into E. coli MM294 to SuR and subsequently
transformants were screened for kanamycin sensitivity.
The transformants were screened for the presence of the
desired 10.2 kb plasmid, pCMC25, which contains a
30 polylinker region proximal to the LB sequences.

            G.1.c. Completion of pCMC90
            The desired pCMC90 was constructed as
follows: pCMC25 was digested with with EcoRI and SalI

-46-

to open the vector next t the polylinker region. pCMC80
was digested with EcoRI and SalI to release the mtAPH-I
expression cassette which was purified and ligated with
the opened pCMC25 vector. The ligation mixture was used
5 to transform E. coli MM294 to Su^R, and Amp^S
transformants were screened for the presence of the
desired 12.9 kb expression carrier plasmid, pCMC90.
                To summarize, pCMC90 is a multicopy, Su^R
Amp^S derivative of the wide host range plasmid, RSF1010,
10 which contains an expression cassette for the modified
mtAPH-I sequence bordered by the T-DNA right and left
border sequences. The inserted cassette contains a
unique SalI site at its 3' end between the
polyadenylation signal and the LB sequence.

15   G.2. Construction of pCMC91
            To obtain pCMC91, pCMC90 was digested with
HindIII and SalI, and the approximately 11.5 kb vetor
fragment was purified. pCMC71 was digested with HindIII
and SalI, and the 1.3 kb fragment containing the APH-II
20 coding region and NOS termination region was also
purified. The 11.5 kb vector and 1.3 kb APH-II fragments
were then ligated and transformed into E. coli MM294 to
Su^R. The correct construction of pCMC91 was confirmed by
restriction analysis and pCMC91 was deposited with ATCC
25 on or about 10 April 1985 and has accession no.

_____.

            G.3. Construction of pCMC92
            pCMC92 was constructed in a manner exactly
analogous to that described for pCMC91 except that pCMC72
30 was used instead of pCMC71 as the source of the APH-II
gene. Thus, pCMC92 has a modified 5' untranslated region
for the APH-II gene lacking the out of frame ATG and the

-47-

bacterial ribosome binding site. pCMC92 was deposited
with ATCC on or about 10 April 1985 and has accession no.

_____.

G.4. Construction of Double-Cassette, Broad
5   Host Range-T-DNA-Bordered Expression
           Carrier Vectors
     pCMC112 contains a T-DNA bordered double
expression cassette for APH-II and β-IFN on a vector
with a broad host range replicon. It was prepared by
10 digesting pCMC91 with SalI, treating with BAP, and
ligating the linearized DNA with the 1.1 kb fragment
isolated after XhoI/SalI digestion of pCMC102. The
ligation mixture was transformed into E. coli MM294, and
SuR colonies screened for the desired 13.8 kb pCMC112.
15 It contains a unique SalI site between the IFN cassette
and the LB sequence.
     In a precisely similar manner, XhoI/SalI
expression cassettes were excised from the expression
carrier vectors, pCMC70 (paragraph E.1), pCMC103
20 (paragraph E.4), and pCMC121 (paragraph E.5), and ligated
into the SalI site of pCMC91. Thus were produced the
following double cassette expression carrier vectors
adaptable to transformation mediated by Agrobacterium:

  pCMC114 containing the APH-II and mtAPH-I cassettes;
25 pCMC113 containing the APH-II and CAT cassettes;
  pCMC123 containing the APH-II and Bt-toxin cassettes.

pCMC123 was deposited with ATCC on or about 10 April 1985
and has accession no. _____.

In all of the foregoing, the cassette series is framed by
30 right border and left border T-DNA sequences, resides on

a plasmid containing a broad host range origin of
replication, and in most cases contains a cassette-unique
-(SalI) site between the polyadenylation signal of the
cassette series and the LB sequence. However, as
5 described in B.2, the opposite orientation of the donor
cassette will result in the placement of the SalI site
between the expression cassettes.

     Also, an EcoRI(blunt)/SalI cassette can be
isolated from pCMC101 (paragraph E.6) and ligated into
10 SalI/SmaI digested pCMC91. Alternatively, pCMC101 can be
digested with SalI, and this plasmid can be ligated with
SalI digested pCMC91, generating a cointegrate plasmid
(pCMC91/101) in which the entire galactosidase expression
vector is inserted into the vector, pCMC91.

15   The following examples illustrate the use of
the expression carrier vectors prepared above and the
results therefrom. In paragraph H is illustrated the
direct transformation of plant cells using pCMC71,
pCMC91, and pCMC77. Of course, any of the other vectors
20 could as well have been used in the same fashion.

     Paragraph I illustrates the use of pCMC91,
pCMC111, and pCMC123 in _Agrobacterium_-mediated
transformation. All of the vectors whose construction
was illustrated in paragraph G above could as well
25 have been used.

H.   **Direct Transformation Using pCMC71, pCMC91,**
    and pCMC77

     The procedure of this paragraph was used to
transform plant cells with pCMC71, pCMC91, and pCMC77.
30 As used in this paragraph, "expression carrier vector"
refers to each of these three plasmids.

     N. tabacum var. H425 protoplasts were prepared
from sterile shoots as described by Krens, et al, _Nature_

-49-

(1982) $\underline{296}$:72, and suspended in a K_3 medium containing
0.4 M sucrose, naphthalene acetic acid (NAA) at 0.1 mg/l
and kinetin at 0.2 mg/l. The protoplasts (5 x 10^5 cells
in 1 ml) were combined with 0.5 ml of 40% (w/v)
5 polyethylene glycol 6000 dissolved in an uptake buffer
(UB) consisting of 140 mM NaCl, 5 mM NaCl, 5 mM KCl, 0.75
mM Na_2PO_4, 5 mM glucose and 125 mM $CaCl_2 \cdot 2H_2O$ at pH
7.0. To this mixture was added 50 µl solution containing
1 to 10 µg expression carrier vector and 0 to 50 µg of
10 purified $\underline{N. \ tabacum}$ var. H425 DNA, added to protect
against degradation. (Higher rates of transformation
were observed in the presence of the purified plant DNA.)
       The protoplasts were incubated at 25°C for 30
min with very gentle shaking. At 5 min intervals
15 thereafter, 2 ml aliquots of the UB medium were added
dropwise to the solution, until a total of 10 ml new UB
medium had been added. The protoplasts were pelleted by
low speed centrifugation and the supernatant was removed.
The pellet was resuspended in 10 ml K_3 medium and plated
20 on sterile Whatman #2 (qualitative) filter paper discs,
and separated from a feeder layer of wild type $\underline{N. \ tabacum}$
var. H425 suspension cells by an additional layer of
filter paper. The cells were grown on a standard M3
nutrient medium (Marton, et al, $\underline{Nature}$ (1979) $\underline{277}$:129)
25 containing phytohormones.
       Cells were plated at a density of 10^2 to 10^4
per 10 cm Petri plate. The cells were incubated in the
dark for 24 hr and then grown for 2 weeks at 2,000 lux in
12 hr photoperiods at 25°C. Cell survival was
30 approximately 50% after this time period. The upper
filter layer on which small cell clumps were grown was
then transferred to M3 medium supplemented with
phytohormones (0.1 mg/l NAA and 0.2 mg/l kinetin) and
G418 (25 mg/l) or kanamycin (100 mg/l) and incubation

-50-

was continued at 25°C with 12 hr photoperiods.
Alternatively, transformed cells were plated at a density
of 10^5/ml in T-75 culture flasks. Cells grow effectively
into individual small clumps in two weeks, and such calli
5 were plated at desired densities onto selective media.
Calli resistant to the antibiotic continued to expand,
while those apparently not transformed by plasmid marker
DNA APH-II ceased growth and gradually turned brown over
the next 2 to 4 weeks.
10   When the calli grown under the above conditions
had reached approximately 1 mm in diameter, they were
harvested and regenerated into plantlets as follows:
calli were picked by scalpel point and placed on M3
medium solidified with 0.4% agar containing phytohormones
15 and kanamycin (100 mg/l). When these calli reached 2 mm
or more in diameter, small pieces were transferred to M3
medium containing 0.1 mg/l NAA and 0.5 mg/l to 1 mg/l
kinetin to induce shoot formation. Shoots were then
excised and placed in rooting medium (1/10 strength M3
20 medium without sucrose or phytohormones, 0.4 mg/l
thiamine and 1% agar, pH 7.9, as described by Barton, K.,
et al, _Cell_ (1983) _32_:1033). Rooted plantlets were
placed in soil under high humidity for several days,
followed by routine greenhouse growth conditions.

25   H.1 Results from Transformation with
                pCMC71

                H.1.a. Insertion of DNA into
                        the Genome
                High molecular weight DNA is isolated from
30 young leaves of plants prepared as in paragraph H as
described by Chilton, M. D., et al, _Nature_ (1982)
_295_:432. Following banding of the DNA in CsCl-ethidium

-51-

bromide gradients, dye is extracted with isopropyl
alcohol equilibrated with 3M NaCl, 0.3 M sodium citrate.
The clear aqueous phase is diluted with 3 volumes of 10
mM Tris-Cl, pH 7.4, 1 mM EDTA, and DNA precipitated by
5 the addition of absolute ethanol to a final concentration
of 70%.

      DNA from transformed and non-transformed
plant cells and from cassette vector pCMC71 (as a
control) are digested to completion with HindIII and
10 SalI. Standard pCMC71 digests are carried out in the
presence of 5 µg calf thymus DNA per well as carrier.
The digested DNAs are loaded into 1x8x8 mm wells in a
horizontal 0.6% agarose gel prepared in Tris-acetate
buffer (Chilton, M. D., et al, _Cell_ (1977) _11_:263).
15 Agarose-gel electrophoresis and transfer of gel-
fractionated DNA to nitrocellulose are carried out as
described in Thomashaw, et al, _Proc Natl Acad Sci (USA)_
(1980) _77_:6448. Plant DNA samples are loaded at 5 µg per
well, and the pCMC71 DNA at various concentrations
20 corresponding to 1 to 100 genome equivalents.

      Hybridization probes are generated by
nick-translation of purified HindIII/SalI 1.3 kb DNA
fragment from plasmid pCMC71, according to the general
method of Maniatis, T., et al, _Proc Natl Acad Sci (USA)_
25 (1975) _72_:1184. The probe is used at a specific activity
of approximately 10^8 cpm/µg DNA for Southern blot
analysis of nitrocellulose-bound DNA fractions (Chirgwin,
T., et al, _Biochemistry_ (1979) _18_:5294). Results of the
Southern analyses show the presence of a HindIII/SalI
30 1.3 kb DNA gene fragment in DNA from regenerated,
transformed plants, corresponding in size to the DNA
fragment from pCMC71 which encodes the APH-II protein.
No similarly sized DNA fragment is observed in the DNA of
normal (untransformed) tobacco plants.

-52-

H.1.b. Transcription of pCMC71

Total RNA fractions from young transformed
and normal plants are isolated according to the method of
Chirgwin (supra), as modified by Bevan, et al, J Mol Appl
5 Genet (1982) 1:539. Leaves and stems of young plants (5
to 50 grams per extraction) are frozen in liquid
nitrogen, powdered, and lyophilized until dry. The
tissue is homogenized in extraction solution containing 4
M guanidine thiocyanate, 25 mM Tris-Cl, pH 7.0, 0.5%
10 sarkosyl and 0.03% Antifoam A Sigma. Sarkosyl is a
trademark for sodium lauryl sarcosinate and was obtained
from Sigma. The homogenate is prepared to a
concentration of 1 gram initial tissue weight/ml
extraction solution. The homogenate is filtered through
15 cheesecloth, followed by centrifugation at 5000 x g for
10 min to remove particulate matter, and the supernatant
liquid adjusted to pH 5.0 with 1 M acetic acid; one
volume absolute ethanol is added to precipitate nucleic
acids. After 2 hr at -20°C, the precipitate is pelleted
20 by centrifugation at 5000 x g for 30 min and redissolved
in 25 ml of 7.5 M guanidine hydrochloride, 25 mM Tris-Cl,
pH 7.0. The pH is adjusted to 5.0 with 1 M acetic acid,
and 18 ml absolute ethanol added. The precipitate which
forms after about 12 hr at -20°C is collected by
25 centrifugation, washed twice with 70% ethanol, and
dissolved in 25 ml of 25 mM EDTA, 0.1% diethyl
pyrocarbonate, pH 8.0. This solution is extracted with
water saturated phenol:chloroform mixture (6:5) and
precipitated with 2 volumes ethanol. The pellet is then
30 resuspended in 8 ml 25 mM Tris-Cl, pH 8.0, containing
0.1% diethylpyrocarbonate. Two ml of 10 M LiCl is added
to precipitate RNA selectively, and the RNA pellet
resulting from centrifugation is fractionated by oligo-dT

-53-

cellulose chromatography (Bantle, et al, <u>Anal Biochem</u>
(1978) <u>72</u>:413.

    Polyadenylated RNA (polyA RNA) is
denatured with 1 M glyoxal at 50°C for 1 hr (McMaster, et
5   al, <u>Proc Natl Acad Sci (USA)</u> (1977) <u>74</u>:4835). The RNA is
electrophoresed and subjected to Northern blot analysis,
as described by Bevan, et al, <u>J Mol Appl Genet</u> (1982)
<u>1</u>:539. Nick translated DNA prepared as in paragraph
H.1.a and labeled to a specific activity of approximately
10 10^8 cpm per μg, is used to probe for APH-II mRNA.
Tobacco ribosomal RNAs are used as molecular weight
standards on gels.

    The pCMC71 probe shows no hybridization to
polyA RNA in normal (untransformed) tobacco, but a
15 hybridization band is observed in mRNA isolated from
plants regenerated from pCMC71-transformed tissue
cultures. The estimated size of the APH-II mRNA is in
excess of 1000 nucleotides.

          H.1.c.   <u>Translation of APH-II in</u>
20   <u>Transformants</u>

    Effective selection for G418 (or
kanamycin) resistance to the transformed plant cells
implies that the introduced gene is expressed. This is
confirmed by showing the presence of APH-II protein in
25 transformed plant cells by immunoassay.

    Tissue samples from the regenerated plants
are grown under conditions of brief radioactive amino
acid labeling with ^3H glycine. Plant supernatant
proteins are extracted by the method of Barton, K., et
30 al, <u>J Biol Chem</u> (1982) <u>257</u>:6089. Supernatant proteins
(500 μg/ml) from either transformed or normal plants, are
mixed with 0.1 volumes of either pre-immune or immune,
anti-APH-II rabbit serum prepared by standard

-54-

procedures. Immunopecipitation is carried out at 37°C
for 4 hr. The immunoprecipitates are pelleted by
centrifugation, dissolved in sodium dodecyl sulfate (SDS)
and analyzed by SDS-PAGE. The radioactive banding
5 pattern was determined by fluorography (Laskey, R. A., et
al, Eur J Biochem (1975) 56:335).
                    Minor radioactive bands of low molecular
weight (less than 27,000 daltons) are present in both
normal and transformed plants, using both pre-immune
10 serum as well as anti-APH-II serum. Distinct bands of
radioactivity are observed at approximately 27,000
daltons, corresponding to the apparent molecular weight
of APH-II in the supernatant proteins derived from
transformed cells, but not in the supernatant from
15 untransformed plant tissue. This polypeptide was immuno-
precipitated only with anti-sera prepared against
APH-II.

         H.2 Results of Transformation with
              pCMC91
20   As the coding sequences to be inserted using
pCMC91 are identical to those inserted using pCMC91,
precisely the same methods are employed and the same
results are obtained in confirming the integration of the
DNA into the genome, the transcription of the coding
25 sequence, and the translation of the messenger.

         H.3 Results of Transformation with
              pCMC77
         Successful transformation and integration into
the genomic DNA from the double cassette vector, pCMC77,
30 is confirmed in an analogous manner to the foregoing
procedures for pCMC71. However, in carrying out the
Southern blot analysis according to the procedure of

-55-

paragraph H.l.a, the probe is prepar d by nick-
translating the 0.5 kb HindIII/BglII β-IFN gene fragment
from pCMC102, and the DNA extracted from the transformed
plant cells digested with both EcoRI and SalI. The
5 double digestion releases the β-IFN gene as a 1.1 kb
fragment.

H.4 Integration of Other Genomic Sequences
        Similarly, confirmation of integration of
coding sequences from other vectors of the invention is
10 obtained by constructing nick translated probes for the
coding sequence DNA by suitable digestion of the
expression carrier vector with restriction enzymes, and
using standard Southern blot techniques to detect the
presence of the desired fragment in the correspondingly
15 digested genomic DNA.

I. Agrobacterium Mediated Transformation of
    N. tabacum
        All of the expression carrier vectors of the
invention which contain the T-DNA border sequences, which
20 mediate Agrobacterium assisted transformation, can be
used in the procedures of this paragraph I. Construction
of such vectors is illustrated in paragraph G, above.
The description of this paragraph is set forth in terms
of pCMC91, however, any of the described vectors or other
25 vectors of the invention which contain T-DNA border
sequences and broad host bacterial origins of replication
can be used. The procedure illustrates the use of an
Agrobacterium mutant which contains a disarmed Ti plasmid
to supply the virulence regions. However, alternate
30 means of supplying such sequences, absent the tumor-
producing sequences in the T-DNA region of the Ti
plasmid, such as cotransformation of an "empty"

-56-

<u>Agrobacterium</u> strain with both a disarmed Ti plasmid and
an expression carrier vector of the invention could be
used.

     I.1. <u>Transformation of A. Tumefaciens</u>
5   <u>with pCMC91</u>

     The disarmed Ti vector employed was that
harbored by <u>A. tumefaciens</u> strain LBA4404, available from
Dr. Paul Hooykaas at the University of Lyden (Ooms, G.,
Hooykaas, P. J. J., et al, <u>Gene</u> (1981) <u>14</u>:33). This
10 strain is non-oncogenic as it harbors only pAL4404, an
avirulent deletion mutant derivative of the octopine Ti
plasmid, pTiAch5, which has a Tn904 insert in SmaI
fragment 3A, and has suffered a deletion extending from
this point rightward to SmaI fragment 6. This plasmid
15 thus lacks the entire octopine T-DNA and octopine
metabolism functions, but retains an intact virulence
region on the left side of the T-DNA (See de Framond, A.
J., et al, <u>Biotechnology</u> (May 1983) 262-269; Hoekema, A.,
et al, <u>Nature</u> (1983) <u>303</u>:179). <u>A. tumefaciens</u> harboring
20 both pCMC91 and the disarmed Ti plasmid pAL4404 was
obtained by transforming <u>A. tumefaciens</u> strain LBA4404
with pCMC91 by the freeze-thaw method of Holsters, et al,
<u>Mol Gen Genet</u> (1978) <u>163</u>:181, and selecting for Su^R.

     I.2. <u>Transformation of N. tabacum Stem</u>
25   <u>Segments with Agrobacterium-Harboring</u>
             <u>pCMC91 and the Disarmed Ti Plasmid</u>

     Stems of <u>Nicotiana</u> <u>tabacum</u>, variation Havana
425 (H425) were surface sterilized with 7% commercial
Chlorox and 80% ethanol, then rinsed with sterile
30 distilled water and cut into 1 cm long segments. The
segments were placed basal end up in Petri dishes
containing complete MS medium (Binns, A., et al, <u>Planta</u>

-57-

(1979) 145:365) with hormonal supplements of 2 mg/l
naphthalene acetic acid (NAA) and 0.3 mg/l kinetin. The
basal end of each stem segment was punctured repeatedly
with syringe needles and inoculated with A. tumefaciens
5   strain LBA4404 transformed with pCMC91. After 5 to 8
days incubation of the inoculated stem segments at 25°C,
with 16 hr of light each day, calli developed at various
positions on the stem segments. The callus regions were
removed from the stem segments and transferred to
10 complete MS medium (MS basal medium supplemented with
2.0 mg/l NAA and 0.3 mg/l kinetin). The medium also
contained 200 mg/l carbenicillin and 500 mg/l vancomycin
to kill the inoculating bacteria, and antibiotics to
select for transformed plant cells. The selection of
15 antibiotics routinely included kanamycin (100 mg/l) or
G418 (25 mg/l) although neomycin or lividomycin are also
acceptable alternatives. After three transfers (at
approximately 4-week intervals) of the small clumps of ·
viable cells which were resistant to kanamycin or G418,
20 the calli are sufficiently enriched in transformed cells
relative to non-transformed cells to enable assay for the
presence of heterologous DNA in the cells, transcription
of the heterologous gene, and heterologous-DNA gene
expression.
25   When the calli grown under the above conditions
reach approximately 1 mm in diameter, they are picked by
scalpel point and placed on M3 medium solidified with
0.4% agar containing phytohormones and kanamycin (100
mg/l). Once calli reaches 2 mm or more in diameter,
30 small pieces are transferred to M3 medium containing 0.1
mg/l NAA and 0.5 mg/l to 1 mg/l kinetin to induce shoot
formation. Shoots are then excised and placed in rooting
medium (1/10 strength MS media without sucrose or phyto-
hormones, 0.4 mg/l thiamine and 1% agar, pH 7.0). Rooted

-58-

plantlets are placed in soil under high humidity for
several days, foll wed by r utine greenhouse growth
conditions.

I.3 Co-cultivation Transformation with
5   pCMC91 and Disarmed Ti Plasmid

N. tabacum variation B425 seedlings were grown
under sterile conditions and young leaves were treated to
generate viable protoplasts, as described in paragraph
H.1. The protoplasts were cultured in a K_3 medium
10 supplemented with NAA at 0.1 mg/l and kinetin at 0.2 mg/l
for 24 hr in the dark, followed by 48 hr incubation at
2,000 lux. The cells, with regenerating cell walls, were
mixed with A. tumefaciens LBA4404 transformed with pCMC91
at 10^5 plant cells and 10^7 bacterial cells per ml.
15   The cells were incubated in K_3 medium
supplemented with phytohormones as above for
approximately 24 hours at 20°C at 2,000 lux, after which
time bacteria were removed by repeated washing, with the
final wash in K_3 medium supplemented with phytohormones
20 as above, and 200 mg/l carbenicillin and 250 mg/l
vancomycin. Cells were then placed on Whatman #2 filters
over feeder layers (as described above for single cell
cloning) in M_3 medium containing phytohormones,
carbenicillin at 200 mg/l and vancomycin at 250 mg/l.
25 Plant cell densities were varied between 10^2 and 10^5
cells per 10 cm Petri plates, the higher dilutions giving
rise to colonies resulting from a single cell. Cells
were allowed to form calli for up to 4 weeks on such
feeder plates, until clumps of cells approximately 0.5 mm
30 in diameter were visible. The upper filters were then
transferred to fresh plates of M_3 medium containing
carbenicillin at 200 mg/l and kanamycin at 100 mg/l (or
alternatively G418 at 25 mg/l). Under this

procedure, transformed calli were rapidly selected by
continued growth due to resistance to the antibiotic
kanamycin, while non-transformed calli ceased growth and
turned brown. After calli reached 1 to 2 mm in diameter,
5 the calli are transferred to fresh plates and increased
in number of cells for direct regeneration of plants as
described in paragraph I.2 and I above.

I.4.   Results of Transformation
     Successful transformations are confirmed in
10 precisely the same manner as set forth in paragraphs
H.1.a-H.1.b for the transformation of plant cells with
pCMC71. Of course, the results are identical as well.
Similarly, using the same procedures but the appropriate
probes and antibodies, successful transformations using
15 any gene sequence encoding cassette can be monitored.

     The following plasmids have been deposited at
the American Type Culture Collection, Rockville,
Maryland, U.S.A. (ATCC) under the terms of the Budapest
Treaty on the International Recognition of the Deposit of
20 Microorganisms for the Purposes of Patent Procedure and
Regulations thereunder (Budapest Treaty) and are thus
maintained and made available according to the terms of
the Budapest Treaty. Availability of such strains is not
to be construed as a license to practice the invention in
25 contravention of the rights granted under the authority
of any government in accordance with its patent laws.
     The deposited plasmids have been assigned the
indicated ATCC deposit numbers. The plasmids have also
been deposited with the Master Culture Collection (CMCC)
30 of Cetus Corporation, Emeryville, California, U.S.A., the
assignee of the present application, and assigned the
indicated CMCC deposit numbers:

-60-

Plasmid and Host	CMCC Deposit No.	ATCC Deposit No.
pβltrp3-4-1 in E. coli K-12/MM294	1730	39646
pCMC1 in E. coli K-12/MM294	1985	39641
pCMC15 in E. coli K-12/MM294	2002	39654
5 pDG144 in E. coli K-12/MM294	1960	39579
pDH5425 in E. coli K-12/MC1000	1503	39645
pDG2326 in B. coli K-12/CS412	1577	39600
pSYC823 in E. coli K-12/JM103	2020	39657
pTiT37 in A. tumefaciens A208	1773	39423
10 pCMC123 in E. coli K-12/MM294		
pCMC91 in E. coli K-12/MM294		
pCMC92 in B. coli K-12/MM294		

-61-

<u>Claims</u>

1. An intermediate carrier vector which
comprises a DNA sequence cassette containing, in order,
proceeding 5'-3' in the sense strand:
5   (a)   a first cassette-unique restriction site;
           (b)   a promoter sequence (oriented 5'-3') which
is normally operable in plant cells;
           (c)   a polylinker;
           (d)   a polyadenylation signal (oriented 5'-3')
10 operable in plant cells; and
           (e)   a second cassette-unique restriction site.

2. The vector of claim 1 which further
includes a desired coding sequence contained in the
polylinker of (c) operably linked to the promoter of (b)
15 and the polyadenylation signal of (d).

3. The vector of claim 1 which further
includes a vector sequence comprising a broad host range
bacterial origin of replication.

4. The vector of claim 3 which further
20 includes a T-DNA border sequence proximal to either or
both of the first restriction site of (a) and to the
second restriction site of (e).

5. An expression carrier vector which comprises
an expression cassette containing:
25   (a)   a promoter sequence normally operable in
plant cells, operably linked to
           (b)   a desired coding sequence;

-62-

(c) a polyadenylation signal operable in
plant cells, operably linked to the desired coding
sequence of (b), and

(d) a cassette-unique *restriction site*
5 proximal to a cassette terminus.

6. The vector of claim 5 wherein the
expression cassette has a cassette-unique restriction
site proximal to each cassette terminus.

7. The vector of claim 5 which further
10 includes a vector sequence *comprising a broad host range*
bacterial origin of replication.

8. The vector of claim 7 which further
includes a T-DNA border sequence proximal to either or
both of the expression cassette termini.

15   9. An expression carrier vector which comprises
a DNA sequence cassette containing:

(a) a first promoter sequence *normally*
operable in plant cells, operably linked to

(b) a first desired coding sequence;
20   (c) a first polyadenylation signal operable in
plant cells, operably linked to the first desired coding
sequence;
and at least one additional expression sequence comprising:

(d) a second promoter normally operable in
25 plant cells, operably linked to

(e) a second desired coding sequence;

(f) a second polyadenylation signal operable
in plant cells, operably linked to the desired coding
sequence; and

-63-

(g)   a cassette-unique restriction site proximal to a DNA sequence cassette terminus.

10.   The vector of claim 9 which further includes a vector sequence comprising a broad host range
5 bacterial origin of replication.

11.   The vector of claim 10 which further includes a T-DNA border sequence proximal to either or both of the DNA sequence cassette termini.

12.   An expression cassette operable in
10 transformed plant cell hosts comprising:
          (a)   a first cassette-unique restriction site;
          (b)   a promoter sequence normally operable in plant cells, operably linked to
          (c)   a desired coding sequence;
15   (d)   a polyadenylation signal operable in plant cells, operably linked to the desired coding sequence of (c); and
          (e)   a second cassette-unique restriction site.

13.   An expression carrier vector which
20 comprises an expression cassette containing
          (a)   a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence, and
25   (b)   at the 5' or 3' terminus of the cassette, a cassette-unique restriction site.

-64-

14. An expression carrier vector which
comprises an expression cassette containing
        (a) a promoter normally operable in plant
cells and a polyadenylation signal operable in plant
5 cells, both operably linked to a desired coding sequence,
and
        (b) at the 5' or 3' terminus of the cassette,
a cassette-unique restriction site
        said cassette being framed by T-DNA border
10 sequences,
        wherein the carrier vector contains a
broad host range bacterial origin of replication.

        15. A plant, including the cells and seeds
thereof, transformed with the vector of claim 5.

15   16. A plant, including the cells and seeds
thereof, transformed with the vector of claim 8.

        17. A plant, including the cells and seeds
thereof, transformed with the vector of claim 9.

        18. A plant, including the cells and seeds
20 thereof, transformed with the vector of claim 11.

        19. A method of transforming a plant,
including the cells and seeds thereof, which comprises
directly transforming a culture of host plant cells with
an expression carrier vector comprising a promoter
25 normally operable in plants and a polyadenylation signal
operable in plant cells, both operably linked to a
desired coding sequence.

-65-

20. A method of transforming a plant including the cells and seeds thereof which comprises directly transforming host plant cells with the vector of claim 5.

21. A method of transforming a plant including
5 the cells and seeds thereof which comprises directly transforming host plant cells with the vector of claim 9.

22. A method of transforming plant cells which comprises:
(a) transforming A. tumefaciens with an
10 expression carrier vector which comprises an expression cassette containing a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence, said expression cassette being proximal to at least one T-DNA border sequence; and a vector fragment containing a
15 broad host range bacterial origin of replication;
(b) providing in the A. tumefaciens host a plasmid comprising the virulence region of a Ti plasmid; and
(c) contacting susceptible plant cells with the resulting A. tumefaciens.

20   23. A method of transforming plant cells which comprises contacting A. tumefaciens-susceptible plant cells with cells of A. tumefaciens which contain:
(a) plasmid comprising the virulence region of a Ti plasmid;
(b) the expression carrier vector of claim 8.

25   24. A method according to claim 23 wherein there is a T-DNA sequence at each terminus of the DNA cassette of the expression carrier vector.

25. A method according to claim 23 wherein the
expression carrier vect r comprises a coding sequence
within the DNA sequence cassette which encodes a dominant
selectable marker for transformed plant cells.

5   26.   The method of claim 23 wherein the plasmid
of (a) is a disarmed Ti plasmid.

         27.   A method of transforming plant cells which
comprises infecting or coculturing <u>A. tumefaciens</u>-
susceptible plant cells with cells of <u>A. tumefaciens</u>
10 which contain:
              (a)   a plasmid comprising the virulence region of
a Ti plasmid; and
              (b)   the expression carrier vector of claim 11.

         28.   The method of claim 27 wherein the plasmid
15 of (a) is a disarmed Ti plasmid.

         29.   A method according to claim 27 wherein the
expression carrier vector comprises a coding sequence
within the DNA sequence cassette which encodes a dominant
selectable marker for transformed plant cells.

20   30.   The method of claim 27 wherein the plasmid
of (a) is a disarmed Ti plasmid.

         31.   A method of transforming plant cells which
comprises mixing a suspension of plant cells or
protoplasts thereof with an effective amount of
25 expression carrier vector which vector includes: a
promoter normally operable in plant cells and a
polyadenylation signal operable in plant cells, both
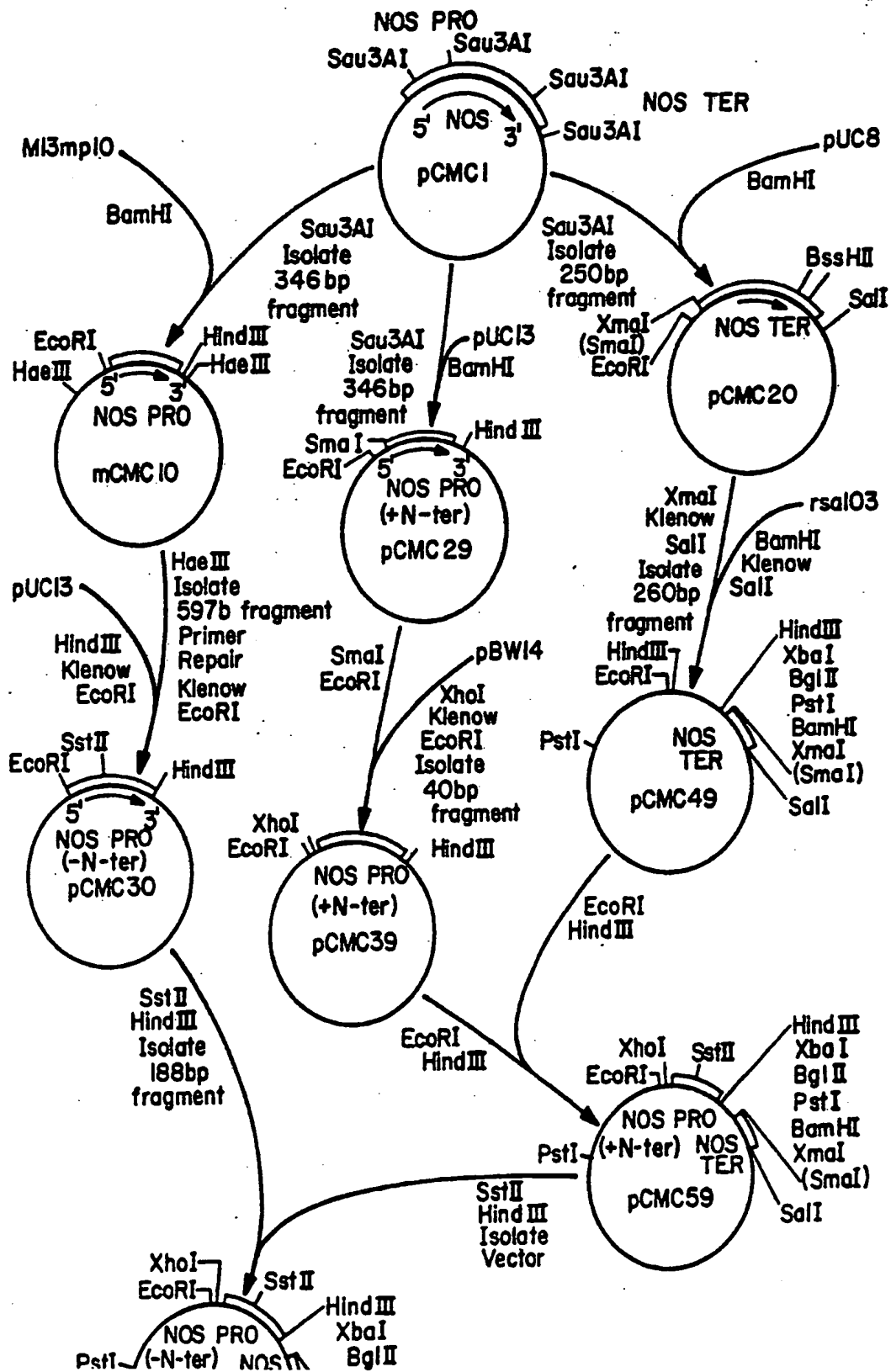operably linked to a desired coding sequence.

-67-

32. A plant, including the cells and seeds thereof transformed by the method of claim 19.

33. A plant, including the cells and seeds thereof transformed by the method of claim 20.

5   34. A plant, including the cells and seeds thereof transformed by the method of claim 21.
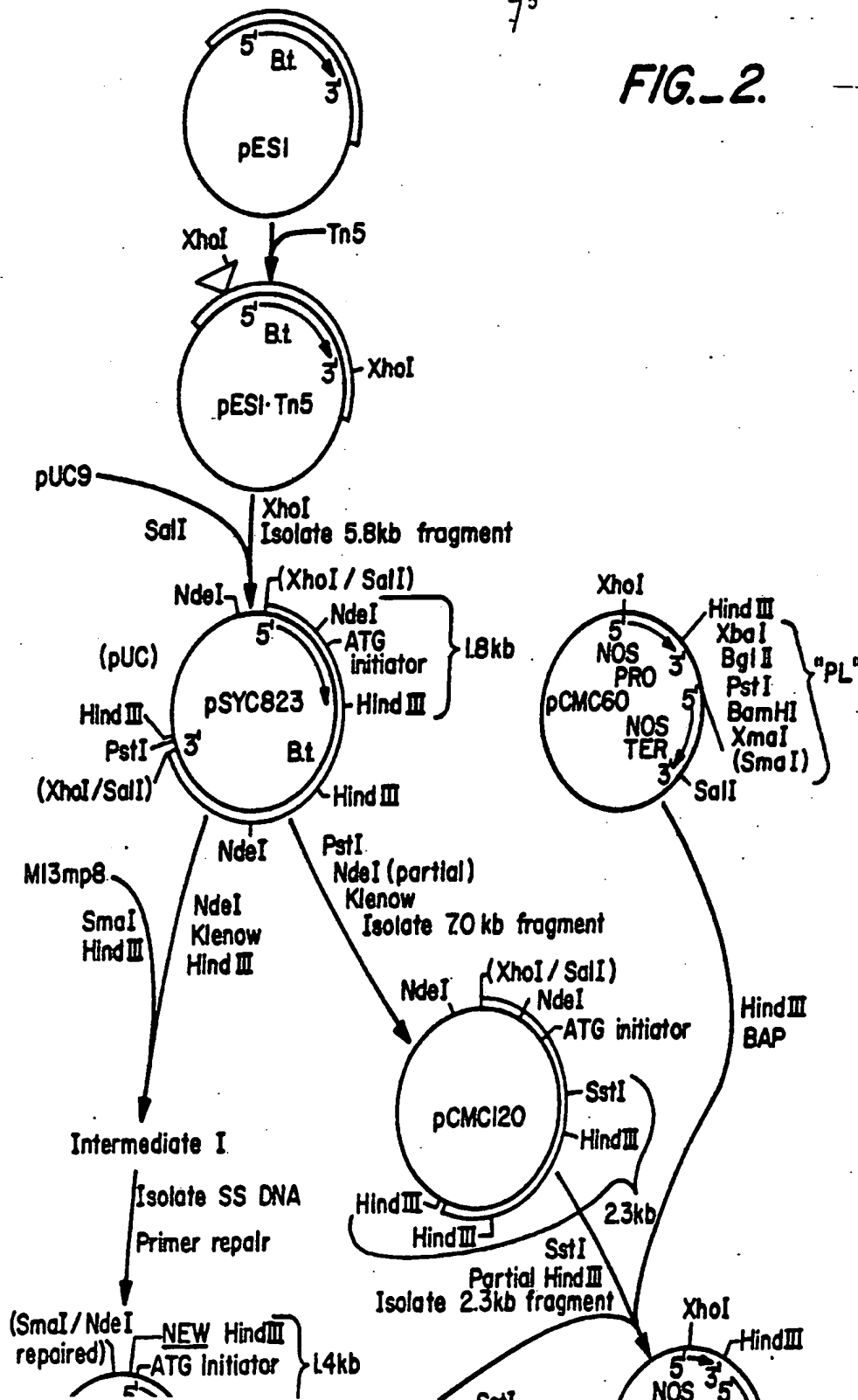
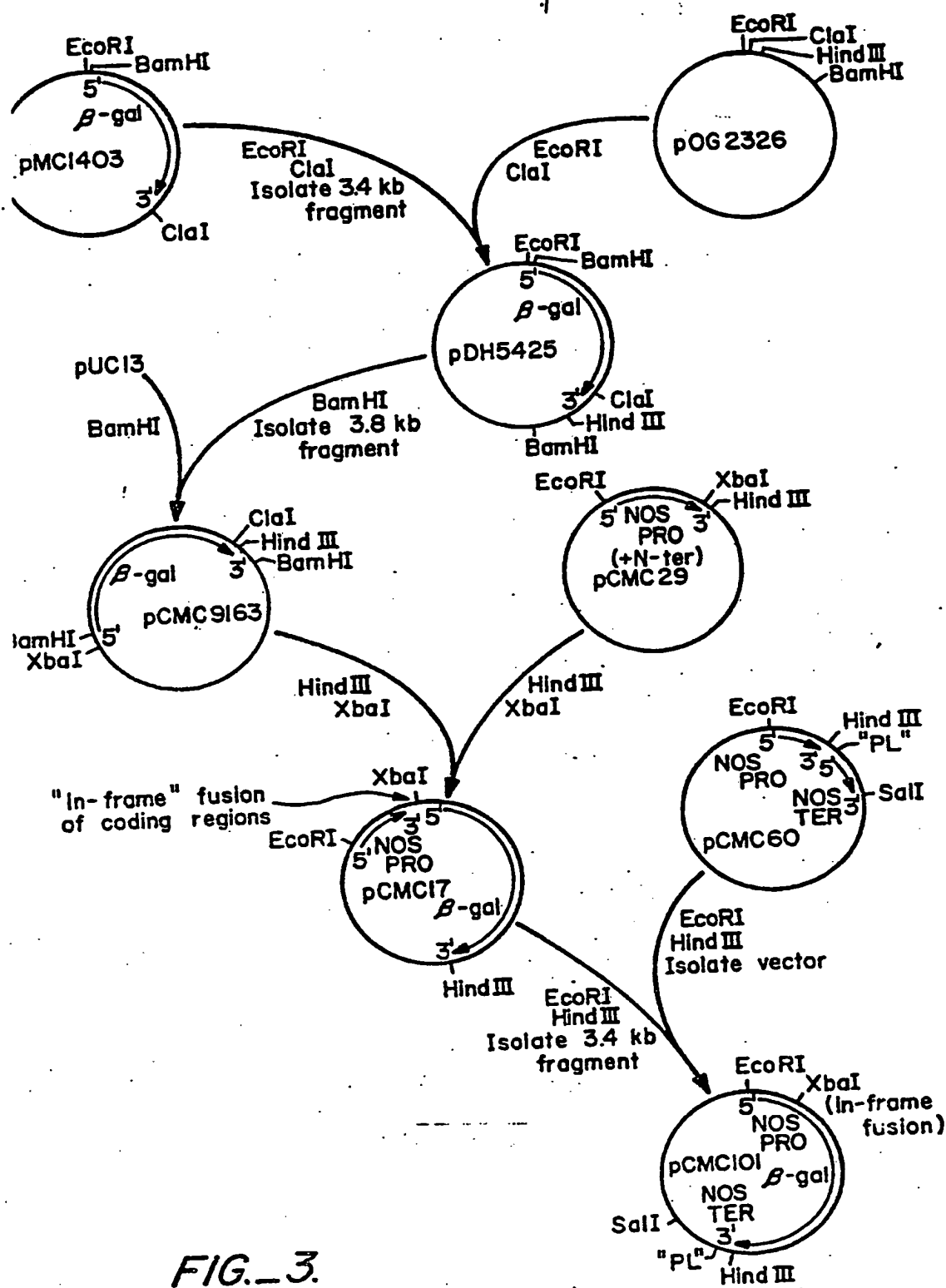35. A plant, including the cells and seeds thereof transformed by the method of claim 23.

36. A plant, including the cells and seeds
10 thereof transformed by the method of claim 27.

FIG._2.

FIG._3.
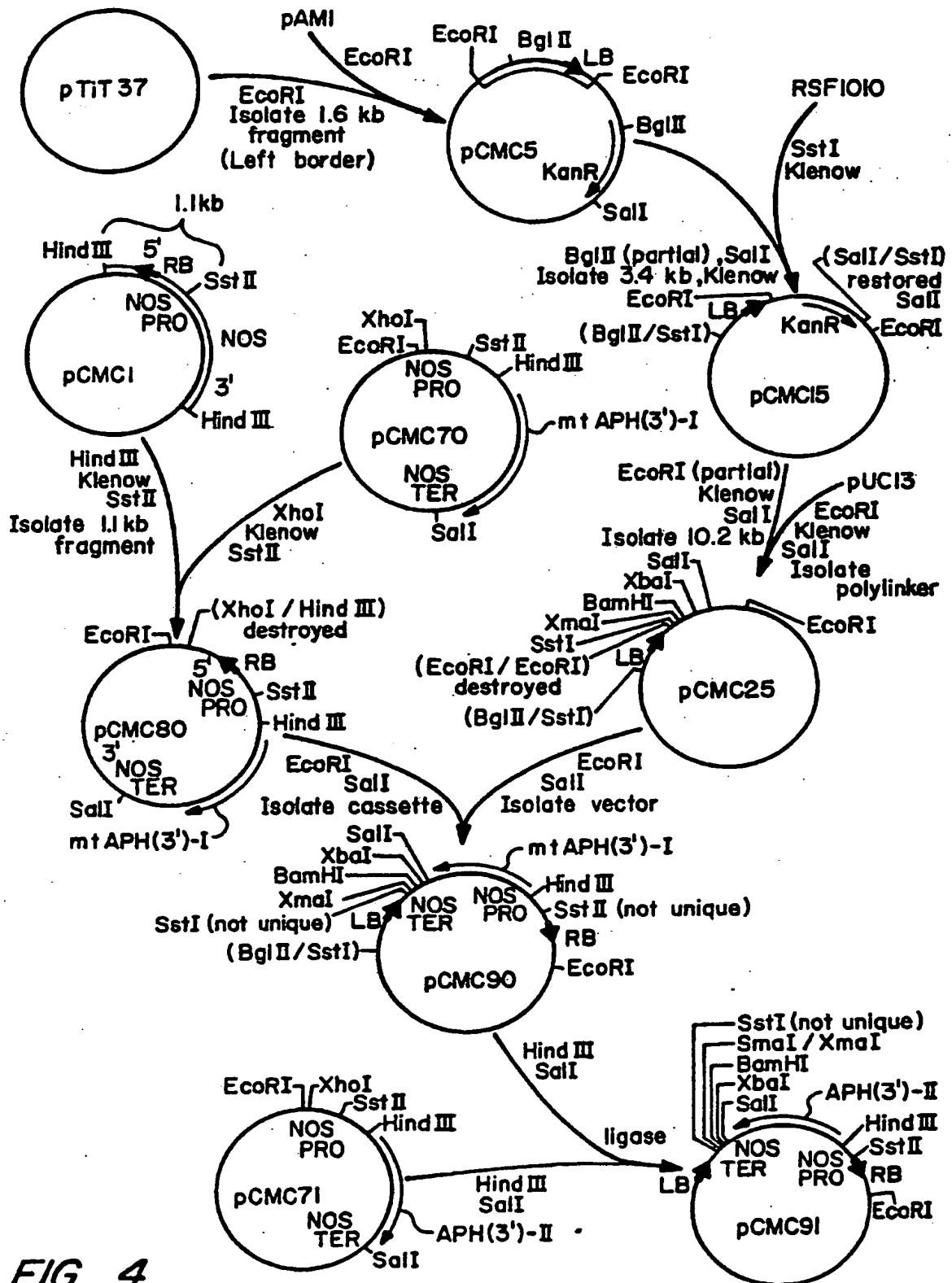
FIG._4.
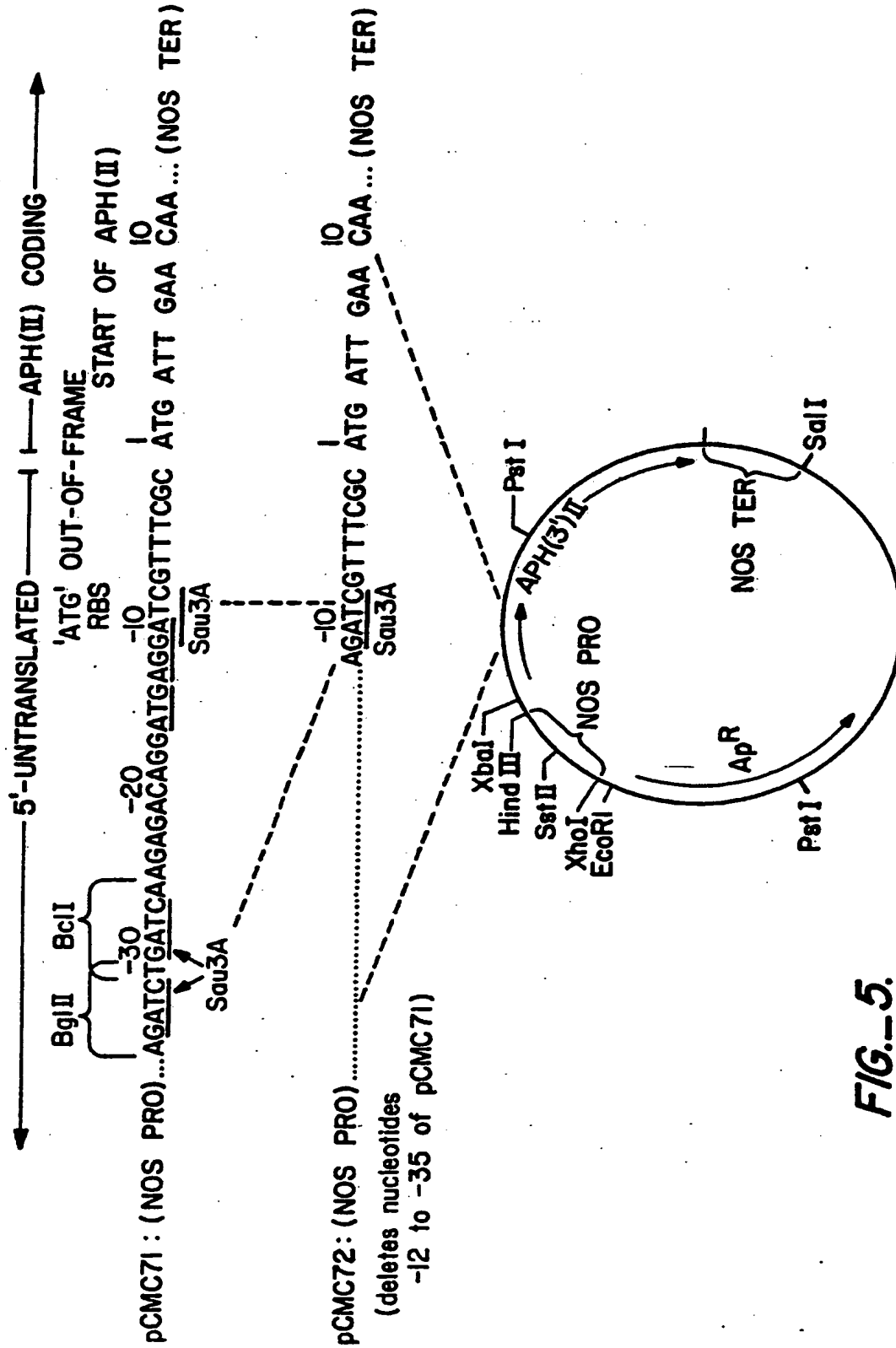
FIG._5.

INTERNATIONAL SEARCH REPORT

International Application No **PCT/US 85/00659**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC[4]: C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N C 12 P

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proceedings of the National Academy of Sciences USA, volume 80, August 1983, R.T. Fraley et al.:"Expression of bacterial genes in plant cells" pages 4803-4807; see the whole document (cited in the application) --	1-14,19-31
Y	The EMBO Journal, volume 2, December 1983, P. Zambryski et al.: "Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity", pages 2143-2150; see the whole document --	1-36
Y	Nature, volume 303, 19 May 1983, L. Herrera-Estrella et al.:"Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector", pages 209-213; see page 210, right hand column and page 211 (cited in the application) --	1-36
A	Bio technology, volume 1, May 1983, A.J. de Framond et al.:	

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th July 1985	0 8 AOUT 1985

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	"Mini-Ti: A new vector strategy for plant genetic engineering", pages 262-269; see figure 6 (cited in the application) --	1-14
A	US, A, 4407956 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 4 October 1983	
P,X	Nucleic Acids Research, volume 12, November 1984, M. Bevan:"Binary Agrobacterium vectors for plant transformation", pages 8711-8721; see figure 2 and page 8717 --	1-14 - 19-31
P,X	WO, A, 84/2919 (MONSANTO CO.) 2 August 1984, see the claims --	1-14, 19-31
P,X	EP, A, 0116718 (MAX-PLANCK-GESELLSCHAFT) 29 August 1984, see the claims --	1-14, 19-31
P,X	EP, A, 0126546 (AGRIGENETICS) 28 November 1984, see claim 1	1-14

III. D CUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

INTERNATIONAL APPLICATION NO.   PCT/US 85/00659 (SA   9397)

This Annex lists the patent family memb rs relating to the
patent documents cited in the above-mentioned international
search report. The members are as contained in the European
Pat nt Office EDP file on 30/07/85

The European Patent Office is in no way liable for these
particulars which are merely given for the purpose of
information.

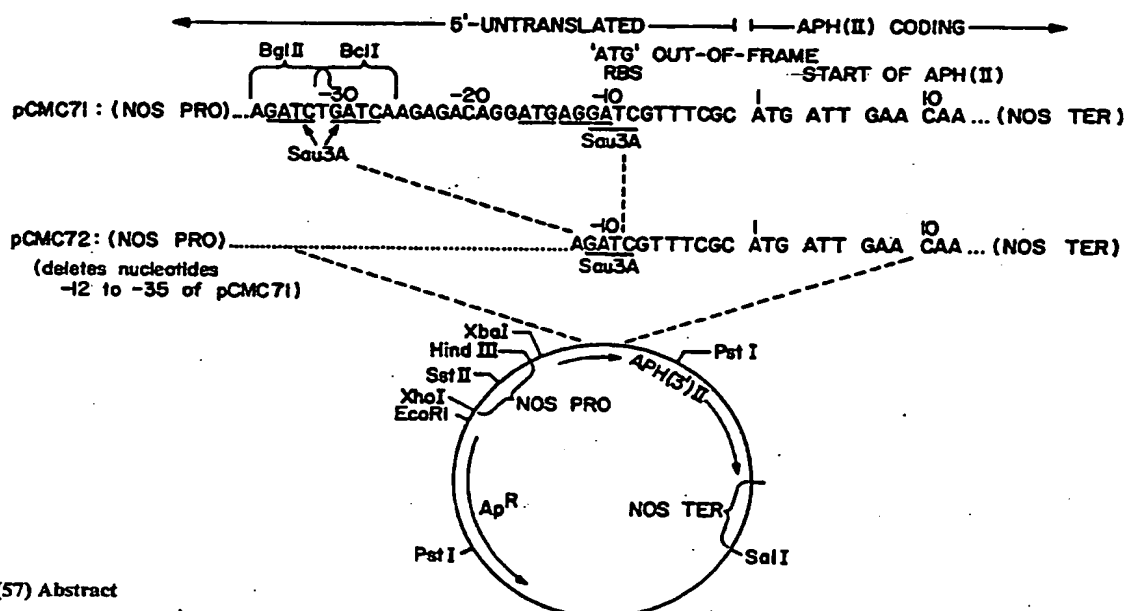| Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A- 4407956	04/10/83	None		
WO-A- 8402919	02/08/84	EP-A- JP-T-	0131624 60500438	23/01/85 04/04/85
EP-A- 0116718	29/08/84	AU-A- JP-A-	2327484 59140885	19/07/84 13/08/84
EP-A- 0126546	28/11/84	AU-A-	2684084	18/10/84

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(74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).

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Published
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(54) Title: METHODS AND VECTORS FOR TRANSFORMATION OF PLANT CELLS

(57) Abstract

Vectors and methods suitable for both direct and *Agrobacterium*-mediated transformation of plants. The vectors comprise easily manipulated expression cassettes and optionally contain border sequences associated with *Agrobacterium* plasmid DNA transfer. By using an intermediate carrier vector which contains the requisite expression control sequences in accessible form, a wide variety of single and multiple cassette vectors can be constructed.

* (Referred to in PCT Gazette No. 05/1986, Section II)